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STUDIES ON ENZYME ACTION.

XXXVIII. THE ESTER-HYDROLYZING ACTIONS OF THE WHOLE EEL.

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(Accepted for publication, June 11, 1926.)

The systematic study of the ester-hydrolyzing enzymes of various animal materials has been described in previous communications.[1] Results obtained with the eel are presented in this paper.

Experimental Methods.

Adult eels whose weights ranged from 86 gm. to 337 gm. were used. Immediately after being killed, they were passed twice through a meat chopper. If the solid material was to be tested directly, 0.67 gm. portions were weighed into flasks and 15 cc. water and 1 cc. toluene added to each. If extracts were to be tested, the requisite amounts of water or solution were added to the weighed solid, allowed to extract overnight at room temperature, filtered through paper, suitably diluted, and 15 cc. portions used for the enzyme tests. In every enzyme test the concentration of the eel, in the form of original solid, or of the eel extract corresponded to 44.4 mg. of original material per cc. of mixture or of solution tested. Because of the increases in volume in the dialysis experiments it was necessary to plan for suitable adjustment of the concentrations of the original extracts. Toluene was present throughout in every experiment.

Dialysis was carried out in collodion bags at 14–17° for 15 to 24 hours, either against tap water where the flow was comparatively rapid and the outside liquid not recovered, or against definite volumes of distilled water with constant stirring and where the outside liquid was used for enzyme tests.

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[1] *Cf.* especially *J. Biol. Chem.*, 1924, lix, 183, 213; 1924–25, lxii, 687, 697; *J. Gen. Physiol.*, 1925–26, viii, 75; 1925–26, ix, 651; *J. Am. Chem. Soc.*, 1924, xlvi, 1885; *J. Cancer Research*, 1925, ix, 105; 1926, x, 146.

The enzyme tests were carried out as described in previous communications: ten different esters were used, as shown in the figures and in the table, 3.4 milli-equivalents of each ester, incubation for 22 hours at 37–38°, titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator, and blanks and duplicates in every case.

Results.

The relative hydrolyzing actions of the solid and different extracts on the ten different esters are shown in Figs. 1 and 2. The averages of three closely agreeing series of experiments are given in each curve, the actions on phenyl acetate being largest and placed equal to 100 in each case.

The curves of the relative actions of the eel solids and extracts showed no striking differences either when tested directly or after dialysis against tap water. Minor differences, however, were indicated in the two figures.

The absolute enzyme actions (actual titration values corrected for blanks), are not given in detail for these experiments. Only the general behavior will be stated: The solid material gave the largest actions, the undialyzed aqueous and 50 per cent glycerol extracts gave much the same actions but somewhat less than the solid, while the undialyzed 10 per cent sodium chloride extracts gave the smallest actions. On dialysis, the absolute actions of the various extracts decreased except for the 10 per cent sodium chloride extracts where the actions on phenyl acetate, glyceryl triacetate, and methyl butyrate were larger for the dialyzed than for the undialyzed extracts.

Table I shows the results of a complete experiment (Experiment F20) in which the enzyme tests were carried out with the solid eel material and the various extracts directly, on these same mixtures after dialysis against distilled water, on the dialysates, and on the dialyzed mixtures to which the dialysates were added. The concentration of each mixture tested was equivalent to 44.4 mg. of original material per cc. of solution tested. The actual titration values, cor-

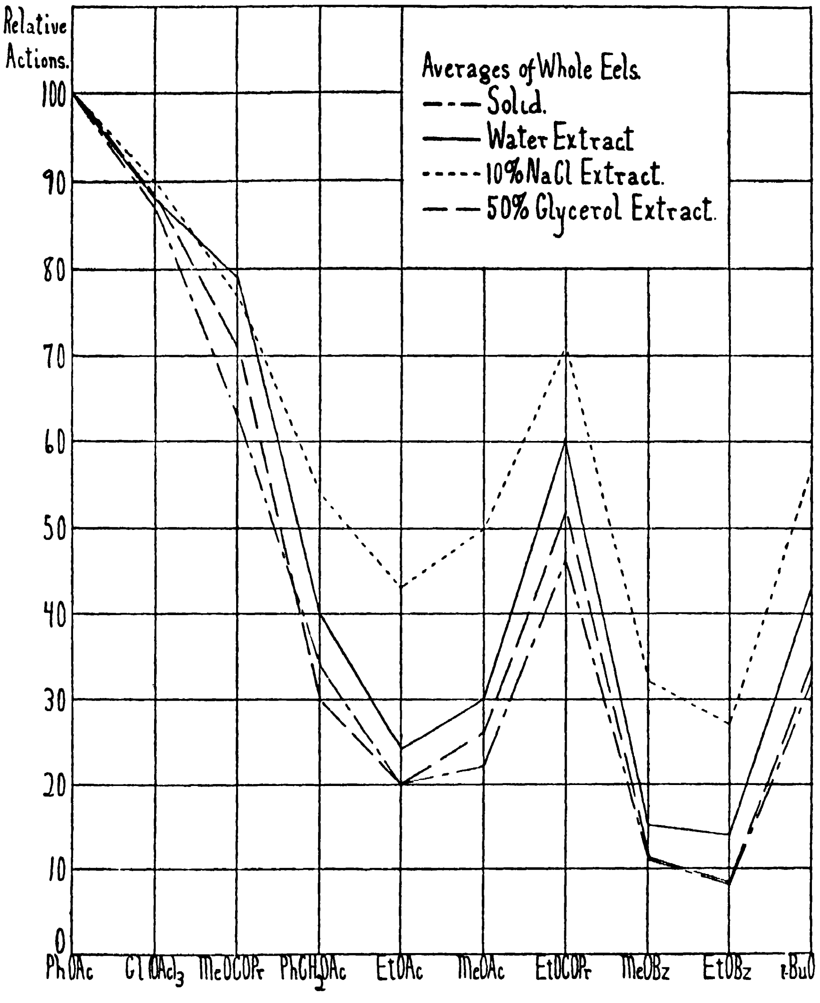

FIG. 1. Averages of relative ester-hydrolyzing actions of solid eel preparation and of undialyzed extracts.

The 10 per cent NaCl extracts were diluted for the tests to contain 3.3 per cent NaCl; the 50 per cent glycerol extracts to contain 16.7 per cent glycerol. The curves of the relative actions of the solids and of the glycerol extracts were essentially the same; compared to these curves the aqueous extracts showed somewhat greater actions on the esters following phenyl acetate and glyceryl triacetate, and the 10 per cent NaCl extracts considerably greater for the last seven esters in comparison with the first three.

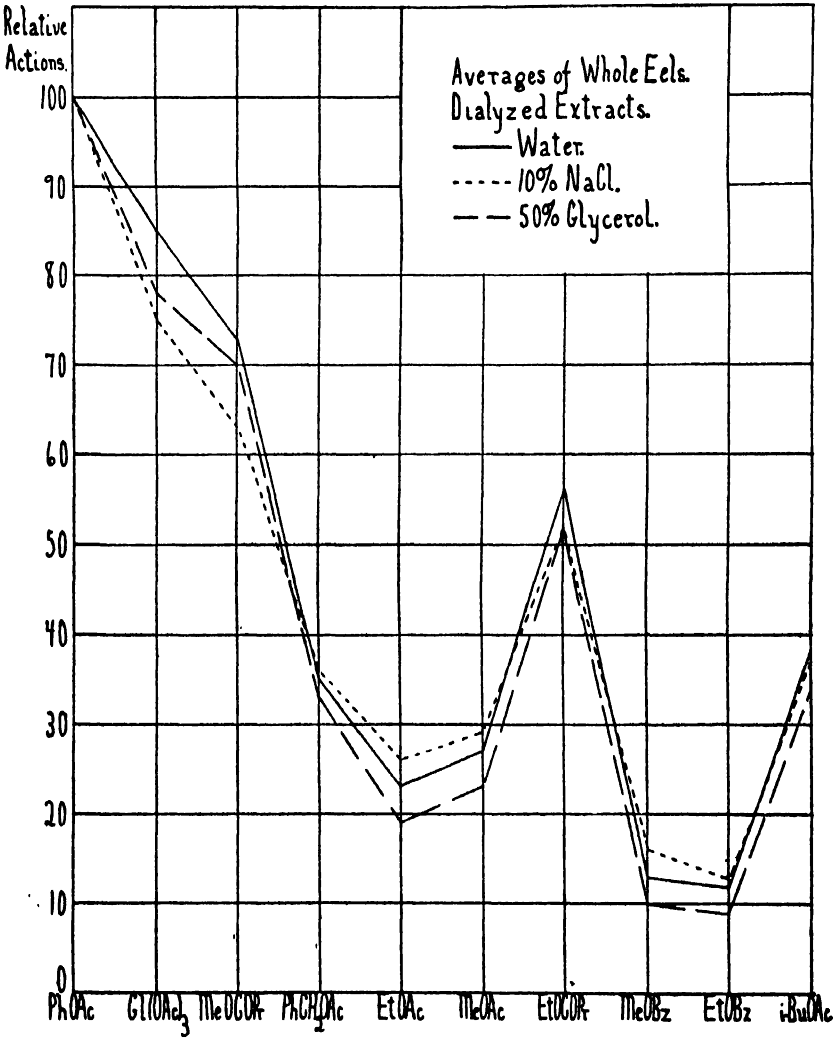

FIG. 2. Averages of relative ester-hydrolyzing actions of eel extracts dialyzed against tap water.

The curves of the three dialyzed extracts were essentially the same. Compared with the undialyzed extracts, the curves for the dialyzed aqueous and 50 per cent glycerol extracts showed no change, while for the 10 per cent NaCl extracts decreased actions relative to that on phenyl acetate were found with the remaining esters for the dialyzed extracts.

rected for blanks, are given. These represent the number of tenths of milli-equivalents of esters hydrolyzed by the enzyme material under the conditions of the tests.

The absolute values are of interest in connection with the curves of relative actions shown in the figures. The figures in italics were calculated from the results of the separate experimental values given in the table.

One striking result is apparent. The calculated sums of the actions of dialysate and dialyzed solution in a number of cases are less than the actions found experimentally when the two were mixed. The dialysates by themselves showed practically no action except on phenyl acetate and glyceryl triacetate and even here the actions were small. The greater actions found experimentally as compared with the calculated include the following: (f) Dialyzed solid plus dialysate for the butyrates, benzyl acetate, and isobutyl acetate; (g) dialyzed solid plus liquid filtered from dialyzed solid plus dialysate for all the esters except phenyl acetate and glyceryl triacetate, but especially the butyrates; (h) liquid filtered from dialyzed solid plus dialysate, especially for the butyrates and isobutyl acetate; (t) dialyzed glycerol extract plus dialysate, small differences for a number of the esters.

These separations of certain of the enzyme materials into two parts, one of which is much less active than the original mixture and the other practically inactive, while the mixture is considerably more active than the sum of the two separate actions, are of interest. Such separations have been reported a number of times with various enzymes and the inactive portion termed "co-enzyme." The results presented here show that the solvent used influences the possibility of the separation and that the action of the co-enzyme may be very different for different substrates. No explanation is at hand for the action of the co-enzyme, but its greater influence on the hydrolysis of the butyric esters may possibly be of significance.

The absolute actions given in Table I supplement the relative actions shown in Figs. 1 and 2.

The ester-hydrolyzing actions of the eel preparations were studied also at 14–17°. It was found that the actions on some of the esters in 22 hours were greater at the lower temperature than at 37–38°. This was shown to be due, in the main, to the combination, or opposing

TABLE I.

Ester-Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced from the Indicated Esters by Eel Preparations.

	PhOAc	$Gl(OAc)_3$	MeOCOPr	$PhCH_2OAc$	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
(a) Original solid.	4.82	4.00	2.86	1.81	0.86	1.02	2.13	0.42	0.41	1.53
(b) Solid-dial. *vs.* dist. water.	2.42	1.90	1.04	0.40	0.25	0.21	0.59	0.13	0.15	0.36
(c) Liquid in bag filtered from solid after dial. *vs.* dist. water.	2.05	1.69	1.49	0.77	0.41	0.50	1.07	0.21	0.19	0.75
(d) Dialysate from solid.	0.53	0.27	0.11	0.04	0.05	0.10	0.04	0.02	0.00	0.02
(e) Solid (b) + liquid (c).	3.88	3.15	2.47	0.99	0.66	0.75	1.60	0.31	0.33	0.97
(e) Solid (b) + liquid (c) (calc.).	*4.47*	*3.59*	*2.53*	*1.17*	*0.66*	*0.71*	*1.66*	*0.34*	*0.34*	*1.31*
(f) Solid (b) + dialysate (d).	2.85	2.26	1.49	0.60	0.32	0.42	1.11	0.27	0.25	0.60
(f) Solid (b) + dialysate (d) (calc.).	*2.95*	*2.17*	*1.15*	*0.44*	*0.30*	*0.31*	*0.63*	*0.15*	*0.15*	*0.38*
(g) Solid (b) + liquid (c) + dialysate (d).	4.29	3.48	3.28	1.55	1.02	1.07	2.26	0.66	0.50	1.49
(g) Solid (b) + liquid (c) + dialysate (d) (calc.).	*5.00*	*3.86*	*2.64*	*1.21*	*0.71*	*0.81*	*1.70*	*0.36*	*0.34*	*1.13*
(h) Liquid (c) + dialysate (d).	2.90	2.41	2.21	1.14	0.66	0.84	1.82	0.46	0.38	1.33
(h) Liquid (c) + dialysate (d) (calc.).	*2.58*	*1.96*	*1.60*	*0.81*	*0.46*	*0.60*	*1.11*	*0.23*	*0.19*	*0.77*
(i) Water extract.	3.66	3.07	2.99	1.53	0.98	1.13	2.35	0.60	0.54	1.57
(j) Water extract dial. *vs.* dist. water.	2.81	2.42	2.06	1.11	0.74	0.84	1.62	0.47	0.42	1.17
(k) Dialysate from water extract.	0.20	0.13	0.02	0.03	0.05	0.08	0.03	0.00	0.02	0.00
(l) Extract (i) + dialysate (k).	3.74	3.20	3.03	1.55	1.00	1.17	2.36	0.73	0.51	1.55
(l) Extract (i) + dialysate (k) (calc.).	*3.86*	*3.20*	*3.01*	*1.56*	*1.03*	*1.21*	*2.38*	*0.60*	*0.56*	*1.57*
(m) Dial. extract (j) + dialysate (k).	3.27	2.75	2.62	1.41	0.85	0.99	2.09	0.66	0.50	1.46
(m) Dial. extract (j) + dialysate (k) (calc.).	*3.01*	*2.55*	*2.08*	*1.14*	*0.79*	*0.92*	*1.65*	*0.47*	*0.44*	*1.17*
(n) 10 per cent NaCl extract.	1.95	1.74	1.49	1.02	0.89	0.96	1.36	0.71	0.56	1.05
(o) 10 per cent NaCl extract dial. *vs.* running water.	2.38	1.87	1.56	0.89	0.66	0.74	1.33	0.45	0.32	0.88

(p) 50 per cent glycerol extract.	3.96	3.25	2.62	1.16	0.76	0.92	1.92	0.41	0.31	1.14
(q) Glycerol extract dial. *vs.* dist. water.	3.04	2.54	2.13	1.05	0.59	0.73	1.63	0.34	0.28	1.08
(r) Dialysate from glycerol extract.	0.57	0.25	0.11	0.03	0.01	0.04	0.06	0.04	0.01	0.00
(s) Extract (p) + dialysate (r).	4.17	3.74	2.65	1.21	1.03	1.09	2.01	0.49	0.31	1.29
(*s*) *Extract* (*p*) + *dialysate* (*r*) (*calc.*).	*4.53*	*3.50*	*2.73*	*1.19*	*0.77*	*0.96*	*1.98*	*0.45*	*0.32*	*1.14*
(t) Dial. extract (q) + dialysate (r).	3.96	3.13	2.41	1.06	0.71	0.88	1.83	0.39	0.35	1.16
(*t*) *Dial. extract* (*q*) + *dialysate* (*r*) (*calc.*).	*3.61*	*2.79*	*2.24*	*1.08*	*0.60*	*0.77*	*1.69*	*0.38*	*0.29*	*1.08*

natures, of two actions; one, the different rates of inactivation of the enzyme at the two temperatures, and the other, the different hydrolytic actions of the enzyme at the same two temperatures. These results, as well as others bearing on the problem of enzyme actions at different temperatures have been presented elsewhere,[2] and will therefore only be referred to in this connection.

SUMMARY.

The hydrolyzing actions of various preparations of the adult eel were studied on ten esters in the usual way. The results are presented in the form of curves for the relative actions and in a table for the absolute actions obtained in one complete experiment.

The separation of the enzyme material in some cases into an active portion and a co-enzyme, the mixture showing greater actions on some esters than the sums of the individual actions, is described and discussed.

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[2] Noyes, H. M., Lorberblatt, I., and Falk, K. G., *J. Biol. Chem.*, 1926, lxviii, 135.

MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

II. The Action of the Chlorides of Lead, Mercury, Copper, Iron, and Aluminum on the Protoplasm of Amœba proteus.

By PAUL REZNIKOFF.

(*From Cornell University Medical College, New York.*)

(Accepted for publication, June 22, 1926.)

The effects on protoplasm of some of the cations found in physiological systems have been reported in a previous communication (1). This study utilized the micrurgical technique and the advantages of such a method over that of simple immersion were pointed out in detail. Only by combining the results of injecting substances into the living cell with those obtained by the immersion method can one obtain proper conceptions of such important physiological problems as those of permeability, site of toxic action, antagonism of ions, and protoplasmic consistency.

Our knowledge of the action of heavy metals has been limited because of the difficulty in localizing the effect of salts in definite parts of the cell. The rapidity with which a cell or tissue reacts has usually been considered to mean ease of penetration (2, 3). But this is not necessarily the case. Even where some change has been noted inside the cell one cannot be certain that it might not be due to a surface effect which involved a secondary change within the interior or that it was not caused by the abstraction of some substance from the interior of the cell.

In a review of the work dealing with the action of disinfectants, especially $HgCl_2$, Cohen (4) points out that previous contributions deal mainly with the course of the process and not with the mechanism concerned. The micrurgical method is especially adapted to a study of the mechanism of the reactions between salts and protoplasm because of the ease with which substances can be brought into direct contact with definite parts of the cells. The importance of many of the metals in certain physiological (5–7) and pathological (8–10) problems suggested their study by means of micrurgy. With the aid of microdissection and injection, therefore, the effects of the chlorides of lead, mercury, copper, iron, and aluminum on *Amœba proteus* were investigated. It is the purpose of this paper to present the results of immersing and tearing amebæ in solutions of these salts and of injecting such solutions into these cells.

The manipulation of the apparatus, the general experimental technique, and the terms used, have been fully described in our former paper (1) and the reader is referred to that report for the details of the procedure. The only general modification of the method used in the experiments described in this paper was that in most of them fewer amebæ were used. This seemed justifiable because of the relatively constant quantitative results with several thousand amebæ from the same stock in former experiments.

I.

Immersion Experiments.

$PbCl_2$.—$PbCl_2$ undergoes gradual hydrolysis in solution, with increase in acidity, and not until a dilution of M/5500 is reached can a solution be maintained at pH 5. If amebæ are immersed in decreasing concentrations of such solutions of $PbCl_2$, they undergo a slow change in shape. They gradually retract their pseudopodia and assume the elliptical form of the so called *Limax* type. The surface is stiffened and the ameba becomes very sluggish. Finally the cell becomes rounded and then dies. The curve labelled $PbCl_2$ in Fig. 1 illustrates the relatively slow action of $PbCl_2$. It is toxic eventually in even very great dilutions. Amebæ cannot survive longer than 5 days until a dilution of M/22,000,000 is reached.

$HgCl_2$.—Amebæ immersed in $HgCl_2$ die very rapidly compared to those in most of the other salts (Fig. 1). In solutions stronger than M/8000 they are converted into small round masses. In solutions ranging from M/8000 to M/250,000 the plasmalemma breaks and the contents begin to scatter but solidify rapidly. Fig. 1 shows that the curve of toxicity for $HgCl_2$ is relatively steep. A solution of M/125,000 is toxic in 1 hour, but amebæ survive more than 5 days in M/500,000. That the immediate effect may not be due entirely to

the acidity which develops when $HgCl_2$ is dissolved in water is shown by the fact that amebæ can live normally in water at pH 6 but die rapidly in the presence of an M/64,000 solution of $HgCl_2$ of the same pH.

$CuCl_2$.—The reaction of an M/1000 solution of $CuCl_2$ is pH 5.5 1 day after the salt is dissolved. After 3 days the acidity increases to pH 4.8. Immersion of amebæ in these solutions of $CuCl_2$ causes the amebæ to become rounded and the contractile vacuole to increase in size. During the first 3 days of immersion amebæ die in a considerable range of dilutions (Fig. 1). In solutions weaker than M/4,100,000 toxicity decreases abruptly.

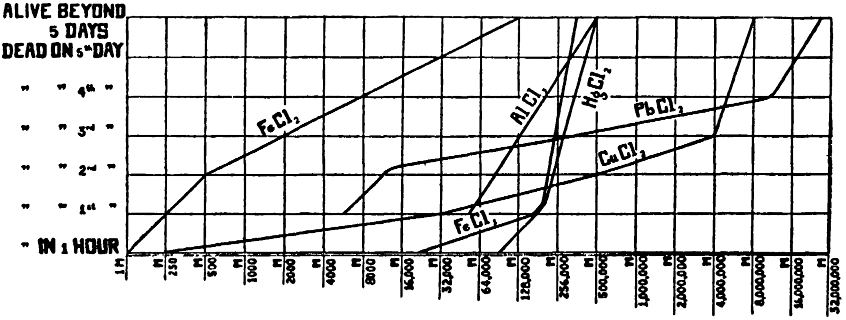

FIG. 1. Viability of amebæ immersed in decreasing concentrations of salt solutions ($PbCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $AlCl_3$).

$FeCl_2$.—The acidity of solutions of $FeCl_2$ increases rapidly on standing, the greatest amount of change, however, occurring early. For example, a solution as dilute as M/66,000 changes from pH 6.2 to pH 5 in 4 days. These solutions of $FeCl_2$ cause immersed amebæ to become rounded and the crystalloid granules within the ameba become blackened. $FeCl_2$ is relatively non-toxic during the first 2 days of immersion after which time the toxicity increases rapidly (Fig. 1). Thus, amebæ can live in a more dilute solution than M/500 for 2 days but cannot live for 5 days until an M/128,000 solution is reached.

$FeCl_3$.[1]—Solutions of $FeCl_3$ gradually increase in acidity (*e. g.* M/325,000 has a reaction of pH 6.2 when first made and is more acid

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[1] In preparing solutions of $FeCl_3$ the molecular weight was taken as 540.44 (Fe_2Cl_6, $12H_2O$).

than pH 5 in 5 days). $FeCl_3$ is much more toxic but produces the same visible effect as the salt of divalent iron. The amebæ become rounded and the crystalloid granules blacken. The maximum toxicity is approached after 1 day of immersion (Fig. 1). Subsequently the curve of toxicity rises more steeply than for any of the other salts used in these experiments.

$AlCl_3$.—Solutions of $AlCl_3$ are acid in reaction over a considerable range (an M/64,000 solution has a pH of 5). This salt, in concentrations of M/400 and higher, causes the amebæ to become round rapidly. In solutions from M/400 to M/32,000 the amebæ continue to move about until their surfaces break. The most striking effect of the salt is the tremendous enlargement of the contractile vacuole (Fig. 2). This occurs in concentrations of M/2,000,000 and stronger. The granules are pushed against the plasmalemma by the distended vacuole. In very dilute solutions, in which the amebæ may live beyond 5 days, the large contractile vacuole may persist for 2 or 3 days but then decreases to its normal size and the ameba recovers completely. The curve of toxicity of $AlCl_3$ is fairly steep (Fig. 1).

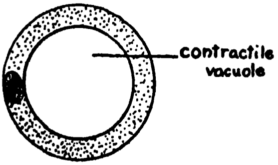

FIG. 2. The effect of $AlCl_3$ on amebæ in injection (M/32 to M/250) and in immersion (M/400 to M/2,000,000) experiments.

Note tremendous enlargement of contractile vacuole.

II.

Injection Experiments.

$PbCl_2$.—In solutions stronger than M/1000, $PbCl_2$ is changed rapidly into the insoluble carbonate by the abstraction of CO_2 from the air. The injection of $PbCl_2$ in concentrations ranging from M/1000 to M/20,000 (Fig. 3) causes the gradual appearance of an irregular, glassy mass containing very few granules. The streaming movements in the rest of the ameba are very active and the glassy mass is

extruded. A second injection immediately after the first results in no solidification. If a large amount is introduced, the surface of the cell may break. If a second injection is made 20 minutes or more after the first, a second solidification and extrusion of the affected region may occur. Subsequent injections have no effect on the viability of the ameba, whether extrusions do or do not occur.

The effect of $PbCl_2$ in causing a delayed coagulation after the first injection and no coagulation after an immediate second injection, suggest that in the reaction between $PbCl_2$ and the protoplasm the lead uses up some cellular constituent which gradually forms anew in the cell. This harmonizes with the suggestion made by Aub and Reznikoff (11) that lead, in the small concentrations used in these experiments, unites with the phosphates or carbonates of the cell. The delay in the formation and the peculiar glassy appearance of the solidified mass in the cytoplasm also point to a different type of reaction between protoplasm and lead than those obtained with the other coagulating ions studied.

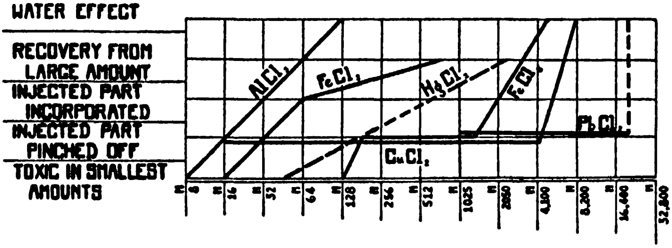

FIG. 3. Recovery of protoplasm of amebæ from injection of decreasing concentrations of $PbCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, and $AlCl_3$. The curve labelled $HgCl_2$ is represented by a broken line because no pinching off of the region injected occurs. The broken line in the curve labelled $PbCl_2$ indicates an abrupt transition from the pinching off of the coagulated region to the water effect. No intermediate stages are seen.

$HgCl_2$.—A solution of M/5 to M/50 $HgCl_2$ causes an immediate solidification of the internal protoplasm (Fig. 3). With solutions of M/100 to M/1600 the surface breaks and some of the contents of the ameba flows out. Small amounts of M/300 $HgCl_2$ cause a disruption of the surface with subsequent recovery. Injections of small amounts of M/600 produce no break and the ameba readily recovers from the

breaks caused by larger injections. A moderate quantity of M/1600 causes no break but the ameba may pinch off the somewhat solidified injected region. An injection of an M/2400 solution even in large quantities causes no disruption of the plasmalemma.

The results indicate that both very high and very low concentrations of $HgCl_2$ act principally on the interior of the cell. Moderate concentrations erode the plasmalemma.

$CuCl_2$.—The injection of solutions of $CuCl_2$ causes a solidification of the affected region and a disintegration of the adjacent surface. With M/16 the ameba can pinch off the affected region (Fig. 3). Stronger solutions solidify the entire cell. Not until M/8000 is reached does this solidification process cease. Each successive injection of $CuCl_2$ in concentrations ranging from M/16 to M/8000 causes the pinching off of the solidified area with its disintegrated surface.

After the solidified area is pinched off, the remnant is apparently normal except for a temporary moderate enlargement of the contractile vacuole. Some enlargement of the contractile vacuole also occurs when very dilute solutions are injected with no resulting local solidification.

$FeCl_2$.—Solutions of $FeCl_2$ stronger than M/32 solidify the internal protoplasm with which they come into direct contact (Fig. 3). With M/32 the affected portion is usually pinched off. M/64 causes a quiescence and partial solidification of the injected region, which is subsequently reincorporated by the active portion of the ameba. Large amounts of M/512 may sometimes cause death, but moderate quantities of M/32 to M/64 as a rule are not fatal. The method by which the ameba constricts off the solidified part when $FeCl_2$ is injected is somewhat different from that observed with most other solidifying agents. Instead of a sharp constriction between the injured and healthy portions (1), the ameba forms a line of demarcation between the healthy and affected area and the living part flows around the solidified region so that the latter lies in a deep depression and is slowly extruded as the depression is everted (Fig. 4). The surface of the extruded region has no definite pellicle in some places. The living portion appears normal except for a slightly enlarged contractile vacuole.

$FeCl_3$.—$FeCl_3$ is much more toxic than the divalent chloride of iron to the internal protoplasm (Fig. 3). A small injection of M/160 solidifies almost the entire ameba and the streaming movements of the unaffected region, which are apparently attempts to pinch itself off, soon cease and the whole cell is killed. From M/320 to M/1280 pinching off usually occurs after each injection. Occasionally, after the introduction of M/320 the ameba may incorporate the affected mass. With M/5120 an extrusion of a small mass from the injected region may occasionally occur after repeated injections. $FeCl_3$ causes the ameba to become sluggish and no typical water effect (1) is obtained until a dilution of M/10,240 is reached. Immediately after injection the vacuole of the ameba enlarges to some extent.

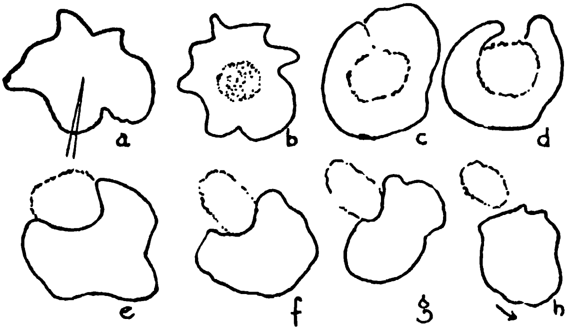

FIG. 4. Injection of M/32 $FeCl_2$ into ameba; *a*, before injection; *b*, solidification of the region injected; *c*, *d*, *e*, *f*, and *g*, extrusion by sliding around solidified area; *h*, complete separation of living remnant from dead area.

$AlCl_3$.—The introduction of $AlCl_3$ has a very striking effect on the ameba. In strengths of M/16 and stronger the injected area is solidified (Fig. 3). M/8 affects the entire ameba but when a solution of M/16 is introduced only the portion injected is solidified and is subsequently pinched off by the unaffected part of the ameba. The injection of M/32 also causes solidification, and pinching off may occur if the injection is made near the edge of the cell. However, in many such cases the ameba may reincorporate the affected mass after it is almost separated from the living portion, as though it were a foreign body. The solidified portion, held by a narrow band is finally en-

gulfed by the ameba and is soon completely absorbed. The various steps of this process are illustrated in Fig. 5. One of the most marked features of the injection of $AlCl_3$ in concentrations ranging from M/32 through M/250 is the tremendous enlargement of the contractile vacuole (Fig. 2). This is identical with the results obtained in the immersion experiments. The ameba recovers from this condition, which may last from a few hours to several days, depending upon the concentration of $AlCl_3$ injected. The similarity in appearance of the internal protoplasm in immersion and injection experiments indicates a high degree of permeability which is in accord with the findings of Michaelis (12).

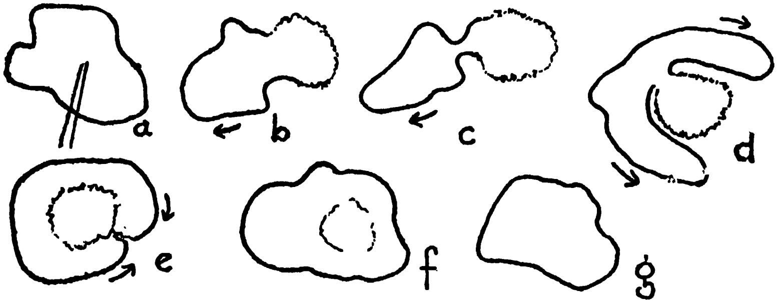

FIG. 5. Injection of M/32 $AlCl_3$ into ameba; *a*, before injection; *b* and *c*, uninjured portion flowing away from injected area and beginning to pinch it off; *d*, almost complete pinching off and beginning of engulfment of injured region; *e*, completion of engulfment; *f*, incorporation; *g*, return to normal state.

III.

Tearing Experiments.

Because of the marked toxicity of even dilute solutions of the heavy metal salts to immersed amebæ, it is very difficult to maintain them alive and in good condition long enough to react visibly to a tearing operation. Thus, in $HgCl_2$ and $FeCl_3$ the rapidity of action of the salts is so great compared to that in salts like $AlCl_3$ and $PbCl_2$ that the amebæ are dead before the needles can be brought into use. Therefore the results of these experiments merely show the dilution at which the immersed amebæ remain alive long enough to react to a marked trauma of the needle. This, of course, varies with the rate of the action of the salt on the plasmalemma.

With this limitation in view the following table indicates the reparability of the torn surfaces of amebæ immersed in these salts.

Salt.	Greatest concentration of salts in which repair of torn plasmalemma takes place.
$AlCl_3$	M/320
$PbCl_2$	M/1,000
$FeCl_2$	M/2,000
$CuCl_2$	M/3,200
$FeCl_3$	M/10,200
$HgCl_2$	M/24,000

DISCUSSION.

These experiments present some evidence as to the actual mechanism involved in the effect on protoplasm of the salts tested. An attempt to group the entire action of all the heavy metals into a common process cannot be justified. There is, however, one common feature which merits consideration. All salts used in this work, $PbCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, and $AlCl_3$, hydrolyze to form strong acids. Moreover, this process of hydrolysis takes place over an extended period of time. This has been studied for lead salts by von Ende (13). Coincident with the increase in acidity the amebæ gradually die and it is probable that this liberation of acid is at least one important factor in their death.

The marked toxicity of these salts when amebæ are immersed in them as compared with the results obtained when the salts are injected into the amebæ indicates that the lethal action of these substances is on the surface of the cell. In no case was the solution sufficiently acid to produce an effect on the internal protoplasm by its acidity alone. This has been shown by experiments previously reported (1), in which repeated injections of a solution of HCl at a reaction of pH 3 had no effect on the internal protoplasm but immersion in HCl at a reaction of pH 5 was lethal in a short time. This lends further support to the view that the action of the salt when injected is due to the cation alone, and that when the ameba is immersed in the solution the effect on the surface is due to the presence of the acid, which is being constantly produced, as well as to the metal cation. It might, however, be suggested that the buffers in a cell can effectively neutralize any acid formed by hydrolysis. But the possibility must be considered that local effects may occur, for example on the surface, and cause irreversible changes before buffering of the acid takes place. This has been suggested by Aub and Reznikoff (11) as a possible mechanism in the action of lead on cells.

That the relative non-toxicity of the salts on the internal protoplasm may be due to their outward diffusion is not probable. No direct evidence for such outward diffusion was found in the case of NaCl (1), and the salts used in these experiments form much more stable compounds with protoplasm than does NaCl.

Some of these salts seem to have an effect on a specific part of the cell, depending upon their concentration. This is particularly true for $HgCl_2$ as has been found also by Bechhold (14) with red blood cells and by MacInnes (15) with *Aspergillus niger.* There is an indication, therefore, that various chemical combinations may be formed between the toxic substance and the different constituents of the cell depending upon the relative concentration of the toxic agent used. Krahé (16) suggests that the action of $HgCl_2$ is due not only to its ionization but, in certain concentrations, to its lipoid solubility.

The gradual increase in toxicity of $FeCl_2$ in the immersion experiments may be associated with its gradual oxidation to the trivalent iron salt which is rapidly toxic. In this connection it is interesting to note that Buschke, Jacobsohn, and Klopstock (17) believe that the "oligodynamic" action of metals depends to a great extent on the ionization of their salts and on an oxidation process.

$PbCl_2$, in the concentrations used in these experiments, probably acts by uniting with the phosphates or carbonates of the cell and thus liberating free acid. Such a secondary reaction is indicated by the slow rate of toxicity.

A striking feature brought out by these experiments is the greater variation in the viability of different amebæ in these solutions when compared to that which occurs in salts such as NaCl, KCl, $CaCl_2$, and $MgCl_2$ (1).

In attempting to determine the mode of action of a toxic substance on a cell it is necessary to consider all the possible mechanisms involved. A toxic agent may (*a*) affect the plasma membrane only, (*b*) affect both the plasma membrane and the internal protoplasm, (*c*) leave the plasma membrane unharmed and injure the internal protoplasm, or (*d*) may not enter the cell but affect it by abstracting a necessary constituent. In considering the third possibility, (*c*), there is no evidence available, so far, to support the belief that a substance may pass through the plasmalemma in a non-toxic form and by some chemical alteration may change into a toxic form inside the cell or may be harmful to the internal protoplasm only. A consideration of the other possibilities, (*a*), (*b*), (*d*), suggests that a substance either abstracts a necessary constituent from the cell or primarily affects its surface. All visible evidence obtained so far points to the fact that toxic agents affect the surface of the immersed cell. So consistent is this result that the suggestion may be made that the maintenance of the surface membrane in a normal state is necessary for the life of the cell.

CONCLUSIONS.

I. *Plasmalemma.*

1. The order of toxicity of the salts used in these experiments on the surface membrane of a cell, taking as a criterion viability of amebæ immersed in solutions for 1 day, is $HgCl_2$, $FeCl_3 > AlCl_3 > CuCl_2 > PbCl_2 > FeCl_2$.

Using viability for 5 days as a criterion, the order of toxicity is $PbCl_2 > CuCl_2 > HgCl_2 > AlCl_3 > FeCl_3 > FeCl_2$.

2. The rate of toxicity is in the order $FeCl_3 > HgCl_2 > AlCl_3 > FeCl_2 > CuCl_2 > PbCl_2$.

3. The ability of amebæ to recover from a marked tear of the plasmalemma in the solutions of the salts occurred in the following order: $AlCl_3 > PbCl_2 > FeCl_2 > CuCl_2 > FeCl_3 > HgCl_2$.

II. *Internal Protoplasm.*

4. The relative toxicity of the salts on the internal protoplasm, judged by the recovery of the amebæ from large injections and the range over which these salts can cause coagulation of the internal protoplasm, is in the following order: $PbCl_2 > CuCl_2 > FeCl_3 > HgCl_2 > FeCl_2 > AlCl_3$.

5. $AlCl_3$ in concentrations between M/32 and M/250 causes a marked temporary enlargement of the contractile vacuole. $FeCl_2$, $FeCl_3$, and $CuCl_3$ produce a slight enlargement of the vacuole.

6. $PbCl_2$, in concentrations used in these experiments, appears to form a different type of combination with the internal protoplasm than do the other salts.

III. *Permeability.*

7. Using the similarity in appearance of the internal protoplasm after injection and after immersion to indicate that the surface is permeable to a substance in which the ameba is immersed, it is concluded that $AlCl_3$ can easily penetrate the intact plasmalemma. $CuCl_2$ also seems to have some penetrating power. None of the other salts studied give visible internal evidence of penetrability into the ameba.

IV. *Toxicity.*

8. The toxic action of the chlorides of the heavy metals used in these experiments, and of aluminum, is exerted principally upon the surface of the cell and is due not only to the action of the metal cation but also to acid which is produced by hydrolysis.

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A SIMPLE ELECTRO-ULTRAFILTER.

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It is frequently desirable to free colloidal solutions of electrolytes, but the process of ordinary dialysis is very slow and often cumbersome. Although the rate of diffusion of electrolytes out of the solution may be increased by means of electrophoresis, the limited choice of membranes available for use with the existing apparatus often leads to unequal rates of passage of the ions to the respective electrodes, and to greater or less marked changes in the hydrogen ion concentration of the solution subjected to dialysis.

We have encountered these difficulties in attempting to dialyze the solutions containing bacteriophage and have been successful in overcoming them by combining the principle of ultrafiltration with electrophoresis.

Our apparatus consists essentially of three concentric chambers, of which the middle one is formed by the collodion membranes deposited on the surfaces of two alundum thimbles (8 and 9, Fig. 1, *a*). The other two chambers serve for removing the dialyzed electrolytes from the membrane by means of a stream of cold water.

The different parts of the dialyzing apparatus are as follows:

(1) A glass tube[1] (3 mm. diameter), supplying distilled water from the reservoir (20) to the inner surface of the alundum thimble (8) carrying the positively charged membrane.

(2) A rubber stopper securing the position of the glass tube (1) in place.

(3) A glass T-tube draining by suction into receptacle (21).

(4) A rubber collar securing an air-tight connection between the T-tube (3) and the carbon (5).

(5) A soft core arc lamp carbon 12 mm. in diameter and 150 mm. long, bored

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[1] This tube is slightly flared out at the lower end and carries a rubber washer to make an air-tight connection at the lower end of the carbon, so that at this point the glass tube is patent, but the hollow carbon (5) is not.

out to admit freely the glass tube (1) and connected with the positive wire of the electric current. 60 mm. from the slightly tapered lower end of the hollow-carbon, a 2 mm. hole permits the return flow of water from the inner chamber around the glass tube (1) and up to the collecting flask (21).

(6) A rubber stopper, No. 13, shelved at its lower end so that a projection 10 mm. deep and approximately 34 mm. in diameter is left to accommodate the outer alundum thimble (9).

(7) A piece of rubber tubing making an air-tight connection between the carbon and the alundum thimble (8).

(8) A Norton alundum thimble, No. 10472 RA 360, 17 mm. in diameter and 80 mm. long. After this part of the apparatus has been assembled, a collodion membrane of the desired degree of permeability is deposited, under pressure, on the outer surface of the thimble (8). For this purpose the T-tube (3) is connected with the vacuum pump, the rubber tube at (1) is clamped off, and the thimble, while under negative pressure, is dipped into the solution of collodion in glacial acetic acid[2] for 60 seconds. The connection with the vacuum pump is now broken, the excess of collodion is allowed to run off, and the coated surface is placed into running warm water to coagulate the collodion and to remove the acid. The removal of the last traces of acid is most quickly accomplished by assembling the entire apparatus and using electrophoresis. The membranes can be used repeatedly, provided they are kept in water when not in use. Their permeability remains practically constant if they are not allowed to dry. To renew the membrane it is necessary to remove the thimble, allow it to dry in air, and when thoroughly dry to incinerate it in the open flame.

(9) Norton alundum thimble, No. 6406 RA 360, 34 mm. in diameter, 100 mm. long. The inner surface of the thimble must be glazed for a distance of 10 mm. from the top, and the collodion membrane that is deposited upon its inner surface ends at this glazed rim. For this purpose, the thimble is fitted by means of a suitably cut rubber stopper into a cylindrical funnel, and attached to the vacuum pump so as to apply negative pressure to the outside of the alundum. The thimble is then filled with collodion for 60 seconds, the collodion is poured off, and warm water used as before. The glazed surface will be found to slip easily over the projecting portion of the rubber stopper (6).

(10) Brass wire gauze, 120 mesh, tightly wrapped around the thimble (9) and held in place by clips connecting it with the lead (12) from the negative pole of the electric circuit.

(11) A glass tube with rubber stopper at each end, carrying the negative lead (12).

(12) Lead from the negative pole of the circuit.

(13) Glass tube carrying distilled water from the reservoir (19) to the middle chamber containing material for dialysis.

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[2] Bechhold, H., and Gutlohn, L., *Z. ang. Chem.*, 1924, xxxvii, 494.

(14) Thermometer.

(15) A glass receptacle serving as an outer chamber and connected with receptacle (21) by a rubber tube forming a movable joint.

(16) Overflow carrying away the ions migrating to the negative electrode.

(17) Stop-cock for emptying glass receptacle (15).

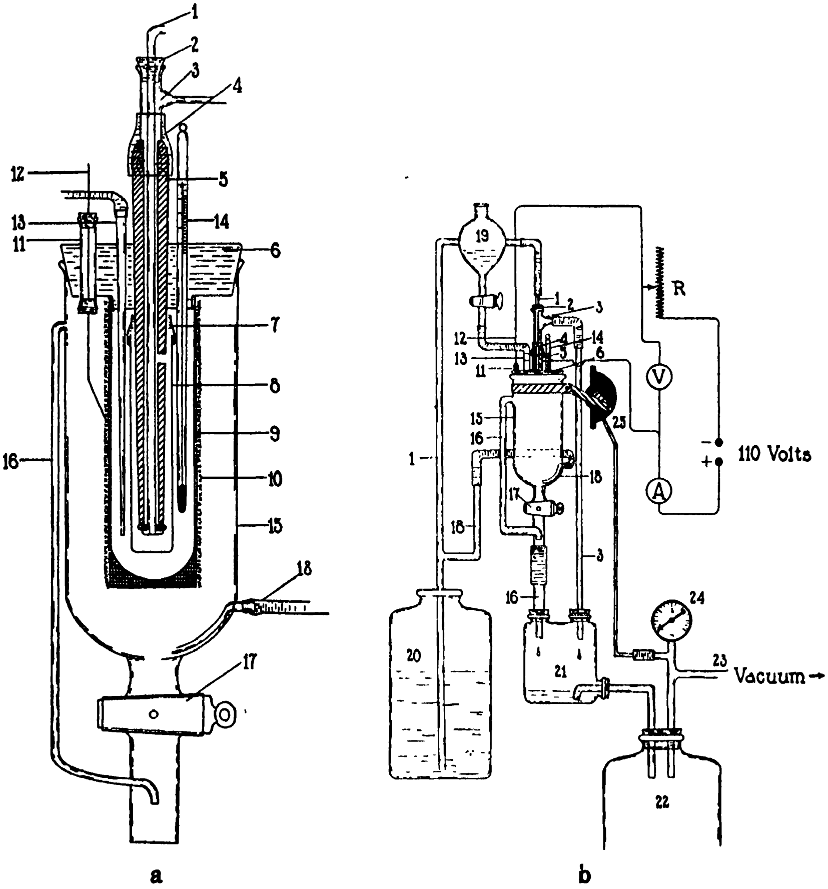

FIG. 1.

(18) Inlet for distilled water.

(19) Fig. 1, *b*. Distilled water reservoir for middle chamber.

(20) Distilled water reservoir for inner and outer chambers.

(21) Intermediary receptacle for dialysates.

(22) Vacuum drainage carboy.

(23) To vacuum pump.

(24) Vacuum gauge.
(25) Vacuum motor (windshield wiper).
(*A*) Weston ammeter (Model 280).
(*V*) Weston voltmeter, with switch (Model 280).
(*R*) Sliding resistance, 650 ohms.

The parts, as well as the assembled apparatus, may be obtained from Eimer and Amend, New York City.

The high efficiency of this apparatus is due to several circumstances. The dialyzing surfaces are very large, considering the total capacity of the apparatus; the relative sizes of the membranes can be varied by using thimbles of appropriate sizes; the permeability of the membranes is easily varied by changing the density of the collodion used in coating the thimbles; the rate of dialysis is speeded up by a constant removal of the dialysate by a constant flow of water at each electrode; the constant flow of cold, distilled water at the electrodes permits the use of high voltage (110–115) without an excessive rise in temperature; the material subjected to dialysis may be kept from becoming concentrated by diluting it during dialysis; the whole dialyzing chamber is agitated, thus preventing the deposit of solids on the membrane. If necessary, the charges on the electrodes may be reversed by using a nickel screen instead of copper, and thus further adjustment in the relative rate of migration of ions may be accomplished.

In the sketch the outflow from both electrodes is mixed in the receptacles (21) and (22). If desirable, it is possible to collect the dialysates separately by leading tube (3) to a separate receptacle analogous tó (21) and similarly connected to the vacuum pump.

The value of combining electrophoresis with ultrafiltration may be seen from the following. When 20 cc. of broth were placed in the dialyzing chamber and the full current was turned on, the ammeter read 4.5 amperes. If this amount of broth were dialyzed without current, it would require 3 hours to increase its resistance to the passage of current sufficiently to give an ammeter reading of 0.016. When dialysis was combined with electrophoresis, a reading of 0.013 amperes was reached in 45 minutes.

INTERPRETATION OF THE LACTATION CURVE.

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INTRODUCTION.

The term lactation curve is used to refer to the curve representing the rate of milk secretion with advance in lactation. It has been known for some years that the group lactation curve for the dairy cow is of a descending exponential type. Sturtevant[1] first published herd data from which he concluded that the milk yield for any month showed a decrease of about 9 per cent as compared with the month preceding. Brody, Ragsdale, and Turner,[2] seeking to correlate the course of milk secretion with the laws governing the rate of chemical reactions, have expressed the lactation curve in the form of an exponential equation, $M_t = M_0 e^{-kt}$, in which M_t is the rate of yield at any time, t, and M_0 is the initial rate of yield. This is the type of equation representing the course of a monomolecular reaction in which M_0 would represent the initial amount of the substance undergoing such reaction and M_t would represent the amount of the substance remaining unchanged at any time, t. This similarity in form of the equations Brody *et al.* interpret in favor of a limiting substance governing the maximum rate of milk secretion at any stage of lactation, the limiting substance undergoing monomolecular change which inactivates it so far as its effect on the rate of milk secretion is concerned.

The above results are based on the raw data of milk yield. It has been shown by Gaines and Davidson[3] that the equation applies even more closely to the data of energy yield than it does to milk yield. While the equation is admirably adapted to describe the lactation curve in simple mathematical terms, the chemical interpretation is open to question. From the viewpoint of Brody *et al.* the factor k of the equation corresponds to the specific velocity constant of the chemical reaction. It is the purpose of the present paper to present some results showing the k values of individual lactation curves as bearing on the interpretation to be placed on the group behavior.[4]

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[2] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1922–23, v, 441.

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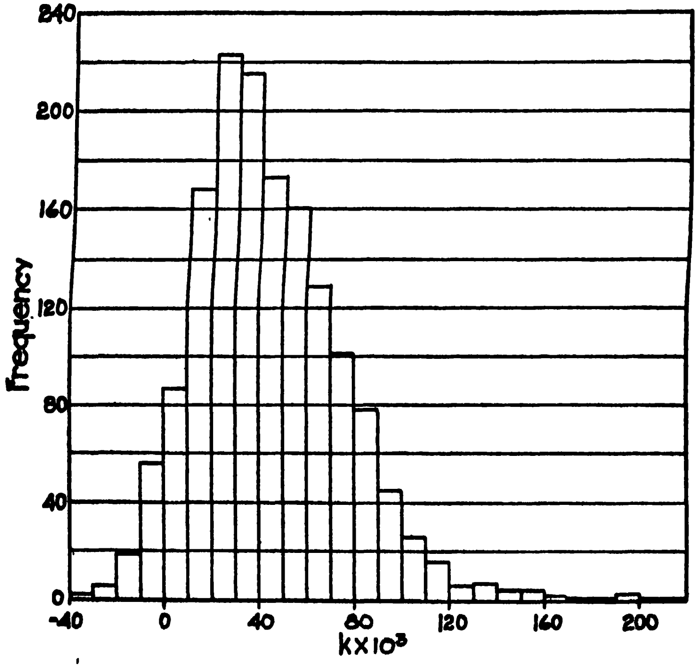

FIG. 1. Frequency distribution of 1534 Guernsey records with respect to rate of decrease per month (k) in rate of yield.

Individual Lactation Curves.

A curve of the type $\frac{dy}{dt} = Ae^{-kt}$ has been fitted by an adaptation of the method previously described to each of 1676 Guernsey records. In the equation y = yield and t = time in months. Yield for a month represents the rate of yield at the middle of that month, with an entirely negligible error. Energy value of the milk solids had been used as being the most fundamental of the several available measures of yield. The distribution of the k values of these records, excluding 142 which were highly irregular, are given in Fig. 1. For a chemical interpretation of the lactation curve we may consider that the data of Fig. 1 represent 1534 determinations of the velocity constant k.

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[4] The data here given are taken from a manuscript presenting a broader investigation of the k values, which has been submitted for publication to the Agricultural Experiment Station.

It will be noted from Fig. 1 that the k values fall into a quite regular order approaching a normal distribution. The mean of the array is .04425 ± .00055; standard deviation, .03219 ± .00089; and coefficient of variability, 72.75 ± .89. There is thus shown a high degree of variability in the k constants.

Ascending Lactation Curves.

It is to be noted further that 83 of the lactation curves, 5.41 per cent of the total, show negative k values, that is, the slope is positive. The velocity constant is not only highly variable, but apparently even reverses its sign in an appreciable number of cases. Obviously some modification of the simple monomolecular interpretation is necessary.

The group behavior of these ascending records is not in good conformity with the equation type, being somewhat aberrant at the start and finish. Indeed, to anyone conversant with the great energy transformations performed by the lactating cow it would be absurd to suppose that the lactation curve could continue to ascend for more than a limited time. The point that any hypothesis must satisfy is that within this group the curve does ascend for 9 or 10 months.

Irregular Lactation Curves.

Under this head are to be considered the 142 records not included in Fig. 1 and necessary to complete a representative sample of the Guernsey records. The average of this group of irregular records is given in Fig. 2 together with 4 individual curves to show the diversity of the records that enter into the average. If the monthly yields are plotted on a logarithmic scale against time on an arithmetic scale, they should fall on a straight line to satisfy the equation. The data are plotted in Fig. 2 to show the degree of conformity to this relation.

The 142 records which we are now considering are so irregular that they were rejected from the main study on the ground that they must have been unduly influenced by extraneous factors. The point of interest in the present connection brought out in Fig. 2 is, that treated as a group we get a result conforming acceptably with the equation. The meaning of the regularity of the group behavior is not clear, when the group is composed of such variable individual elements. It means that the individual deviations from the type curve are of a compensating nature. On the theory of a limiting substance determining the maximum rate of milk secretion, it is easy to account for minus deviations through the effect of other factors in preventing

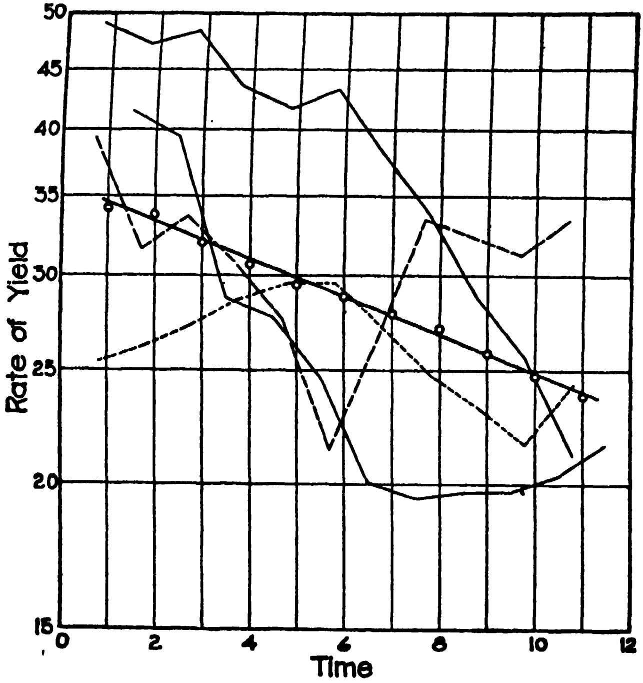

FIG. 2. Showing the average of 142 irregular lactation curves (circles) and 4 individual curves to indicate the diversity. Time is expressed in months from calving. Rate of yield is expressed in pounds of 4 per cent milk per day. One pound 4 per cent milk = 331 large calories.

realization of the maximum, but the difficulty lies in accounting for the plus deviations which serve to counterbalance the minus deviations.

Factors Affecting the Rate of Decrease.

That the k values of the lactation curve equations are greatly affected by conditions of feeding and management of the herd is sufficiently evident from the difference in the performance under advanced registry conditions and conditions of commercial milk production. It may be presumed that the same cows that show under advanced registry a mean k value of .044 would show under the less favorable commercial conditions a mean value of .09 to .1. Obviously nutritional conditions are a powerful factor affecting the rate of decrease.

The k values are also closely related to the initial rate of yield, the correlation between k and A being $r = .535 \pm .012$. On the limiting substance theory the initial quantity of this substance is directly proportional to A. The limiting substance is assumed to disappear in accordance with a monomolecular reaction. The velocity constant of the reaction must be assumed, therefore, to vary directly with the initial amount of the limiting substance, in order to satisfy the observed results. One of the laws of unimolecular reaction is that the velocity constant is the same regardless of the concentration of the reacting substance. Consequently, the observed relation between k and A is in conflict with the theory of a limiting substance and its monomolecular inactivation.

On the other hand, regarded as a nutritional matter and bearing in mind the large energy requirements of lactation, it is not surprising that the rate of decrease should be greater the greater the initial rate of yield.

SUMMARY.

The validity of the assumption of a substance determining the rate of milk secretion and undergoing monomolecular destruction, based on group behavior, is questioned on the evidence from a large number of individual lactation curves. It seems probable that the rate of decrease in the rate of milk secretion with advance in lactation is dependent upon factors of a nutritional nature.

RATE OF RESPIRATION AS RELATED TO AGE.*

BY J. MILTON HOVER AND FELIX G. GUSTAFSON.

(From the Department of Botany of the University of Michigan, Ann Arbor.)

(Accepted for publication, June 21, 1926.)

It is usually stated in the literature that respiration is most rapid in the young and actively growing parts of a plant and decreases with age; and sometimes it is said that respiration conforms to the grand period of growth.

Considerable experimental evidence upholds the first statement. Bonnier and Mangin[1] studied the respiration of several different plants, among them *Evonymus japonica.* Leaves 1 year old respired more per volume of leaf than leaves produced the year of the experiment. Data for other plants were obtained (at different temperatures as far apart as 13 degrees but no corrections were made). M. A. Maige[2] found that most flowers produce more CO_2 per gm. of green weight in the bud stage than when open, but some respire most actively in the open stage. G. Maige[3] found that the intensity of respiration of floral organs decreased with age, except in the pistil, which often showed increased respiration as long as it continued to develop. Nicolas[4] found that of two samples of twigs the younger had a higher rate of respiration. Studying young and adult leaves he obtained the same results. Briggs, Kidd, and West[5] state that, "the evidence available (*Helianthus*) is that the respiration per unit dry-weight of the whole plant at constant temperature decreases with age."

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* Paper from the Department of Botany of the University of Michigan, No. 250.

[1] Bonnier, G., and Mangin, L., Recherches sur les variations de la respiration avec le developpement des plantes, *Ann. sc. nat. bot.*, 1885, ii, series 7, 315.

[2] Maige, M. A., Recherches sur la respiration de la fleur, *Rev. gén. bot.*, 1907, xix, 8.

[3] Maige, G., Recherches sur la respiration des différentes pièces florales, *Ann. sc. nat. bot.*, 1911, xiv, series 9, 1.

[4] Nicolas, G., The variation in the respiration of plants in proportion to age, *Bull. soc. hist. nat. Afrique nord*, 1910, No. 7, 109. (*Exp. Station Rec.*, 1912, xxvi, 628.)

[5] Briggs, G. E., Kidd, F., and West, C., A quantitative analysis of plant growth. Part II, *Ann. Applied Biol.*, 1920–21, vii, 202.

On the other hand A. Mayer,[6] and also Rischawi[7] claim to have demonstrated that the respiration follows the grand period of growth. These investigators enclosed seedling plants in a respiration chamber, and either by daily measurements of the O_2 intake or of the CO_2 given off arrived at the conclusion that there was a grand cycle of respiration conforming to the grand period of growth. In their experiments the normal photosynthetic activity of the plants was prevented and no account was taken of any changes in weight due to the metabolism of the plants. In other words, their results record the respiratory changes in a growing plant independent of any loss or increase in the weight of the plant during its growing period.

The purpose of the present investigation has been to determine carefully the relation between rate of respiration and age. To accomplish this an effort was made to determine the comparative rate of respiration in successive leaves of several plants. In the plants chosen new leaves continue to appear at the top of the plant until the panicles are formed. Before these are formed, however, some of the older leaves at the base have withered away. The successive leaves of these plants, therefore, constitute an age series. In corn the total leaves may number anywhere from 18 to 24, but at any one time there are usually not more than 8 to 12 leaves. In sorghum the total number of leaves developed is 25 to 30 with 8 to 15 active leaves present at one time. In oats and wheat there are only 4 to 6 leaves at any one time. The number of active leaves present appears to be related to age, varietal differences, and water supply. During a dry season the life cycle of a leaf is shorter than during a wet season.

In determining the rate of respiration, the active leaves of the plants were removed and the total CO_2 given off by each leaf was simultaneously determined, by placing the leaves in a battery of Pettenkofer tubes. Inasmuch as the leaves of a plant were all removed at the same time, placed in their respective respiration chambers at the same time, and the CO_2 given off was determined for the same interval, and further, since temperature and other external conditions were identical, it follows that any differences in the rate of respiration are due to differences in the leaves themselves.

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[6] Mayer, A., Ueber den Verlauf der Atmung beim keimenden Weizen, *Landw. Versuchs-Stationen*, 1875, xviii, 245.

[7] Rischawi, L., Einige Versuche über die Atmung der Pflanzen, *Landw. Versuchs-Stationen*, 1876, xix, 321.

The rate of respiration given is the amount of CO_2 given off per gm. of dry matter or per gm. of green weight for the duration of the experiment. Since there were variations in the duration of the experiments and also in the temperature conditions, the rates of respiration in one experiment cannot, except in a general way, be compared with the rates in another. In general, the duration of an experiment was from 18 to 22 hours.

All possible precautions were taken to insure that the air entering the respiration chambers was free from CO_2; care was also taken that all the CO_2 was absorbed by the barium hydroxide tubes. The barium hydroxide after having been standardized was kept in containers entirely free from CO_2.

To illustrate the procedure a typical experiment will be described. A corn plant with ten healthy leaves has been selected in the field. It is brought into the laboratory and the leaves are carefully removed and placed in separate bottles of 500 cc., capacity which are completely covered with black paper. The experiment is arranged as follows: First come 10 $CaCl_2$ towers filled with pieces of soda lime to absorb the CO_2 from the entering air; following each tower is a bottle containing a solution of BaOH, to indicate whether all the CO_2 has been absorbed; to these bottles are attached the respiration chambers, covered with black paper, which in turn are attached to the Pettenkofer tubes, containing 100 to 150 cc. of standardized BaOH (the amount depends on the size of the plant; with large plants more BaOH is used than with small plants); each tube is followed by a bottle containing a solution of BaOH, to insure that all CO_2 has been absorbed by the standardized BaOH; these bottles are connected by Y-tubes to a single aspirator, by means of which the air is drawn through the apparatus. The rate and size of the gas bubbles passing through each tube are regulated so as to be the same in all tubes. Before the experiment is started the respiration bottles are thoroughly freed of all CO_2 by running CO_2-free air through them. The experiment is then run for 22 hours. At that time the BaOH in each tube is titrated with N/10 oxalic acid.

1 cc. of N/10 oxalic acid is equivalent to 0.0022 gm. of CO_2, and from the difference between the original titrations of the standardized BaOH and the titrations at the end of the experiment the amount of CO_2 can readily be calculated.

The amount of CO_2 has been calculated both on the basis of dry and of green weight. The results are similar, and as it is more usual to employ dry weight than green weight, and as the respiration is undoubtedly more closely related to the dry material than to the water of the plant, only the data calculated for dry weight are given. As pointed out before, no two experiments were carried out under identical conditions, nor were the plants themselves identical; some of these experiments were performed during the summer of 1923, others during 1924. For this reason no average of the experiments can be obtained. A few of the experiments have been selected as illustrating the condition in these plants.

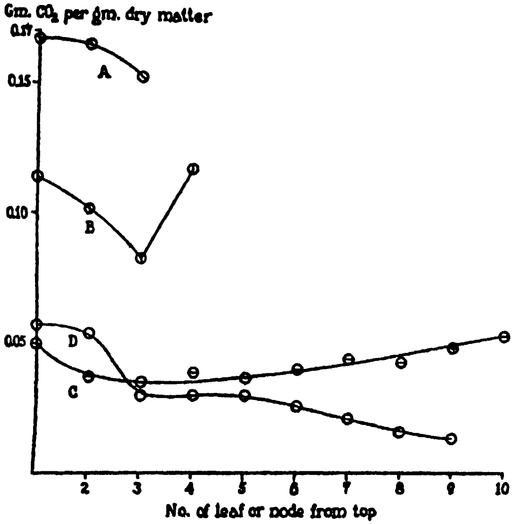

FIG. 1. Curve *A* represents the respiration of 3 corn plants about 4 inches high, with 3 leaves; *B*, 1 corn plant 6 inches high with 4 leaves; *C*, 1 corn plant at the time of pollination with 10 leaves. Curve *D* represents the respiration of a corn stem cut up in such a way that each piece contains one node and part of the contiguous internodes.

Corn (*Zea Mays*) was the original plant worked with and the results of a few of the experiments are given in Fig. 1. Curves *A*, *B*, and *C* represent plants of different ages with varying number of leaves. Curve *A* shows that there is a decrease in rate of respiration with age when the plant is very young, while *B* shows that as the plant ages and the number of leaves increases the rate of the older leaves increases above that of those slightly younger; and *C* (a plant with ten leaves) shows that the oldest leaves may actually respire more than do the youngest leaves on the plant. Curve *D* represents an experiment with the corn stem, which shows that there is a decrease in respiration with increase in age.

In Fig. 2 Curves *A*, *B*, and *C* represent respiration in *Sorghum vulgare*. All of these experiments show that at first there is a decrease in respiration as the leaves age, but that after a certain age has been

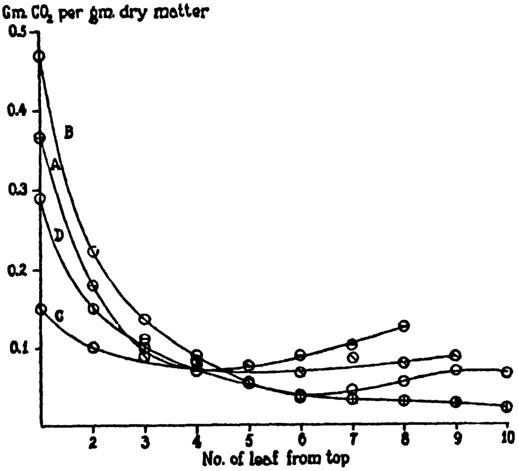

FIG. 2. Curve *A* represents the respiration of 4 sorghum plants 1 foot high with 8 leaves; Curve *B*, 1 sorghum plant 3 feet high with 10 leaves; *C*, 1 sorghum plant developing tassel, with 9 leaves; Curve *D*, sunflower plant about half grown, with 10 leaves.

reached there is a gradual increase in rate, which in some instances is nearly as great as that in the youngest leaves. Curve *D* represents leaves of sunflower (*Helianthus annuus*), and in this as well as in other experiments on sunflower there is a decrease from the first with no subsequent increase.

Fig. 3 represents results obtained with oats (*Avena sativa*). The two experiments were conducted at the same time. These experiments show that the respiration in the oldest leaves is much more rapid than in the youngest.

In Fig. 4 are results of two experiments with nearly mature wheat (*Triticum sativum*). These plants had five leaves but the two oldest ones were dying and so cannot be counted. Both experiments show that the oldest healthy leaves respired more vigorously than the youngest leaves of the plants.

The plants represented in Figs. 3 and 4 were nearly mature and the youngest leaves had already reached the age at which the respiration is at its lowest; all the curves but *B* of Fig. 4 show only increase in respiration with age rather than a decrease followed by an increase.

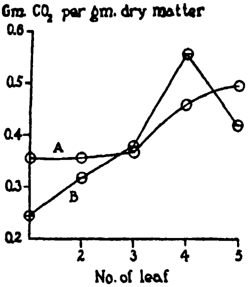

FIG. 3.

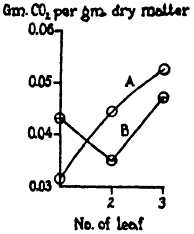

FIG. 4.

FIG. 3. Two oat plants nearly mature. The two experiments were conducted at the same time. These curves strikingly bring out individual differences.

FIG. 4. Two wheat plants, nearly mature; each plant had 5 leaves, but the two lowermost were practically dead. The two experiments were conducted at the same time. They also show individual differences.

It is quite apparent from the data given that when dry weight is taken as a criterion for judging the comparative rate of respiration in the various leaves of corn, sorghum, sunflower, oats, and wheat the respiration cycle does not correspond to the grand period of growth, nor is there a decrease in respiration with age except in the leaves of sunflower and corn stem. The reason other investigators have not noted this before is that not enough controlled experiments have been performed. In most instances a few leaves of one age were taken at one time at a certain temperature and a few leaves of a different age at another time, at a temperature different from the first, without any correction being applied. As far as the writers are aware no experiments have been performed in which a series of leaves or other plant parts, differing in age, have been studied at the same time. It is obvious from the figures given in this paper that, if only two leaves had been taken, in practically all instances the older leaves would have respired less than the very youngest on the plant, but it is equally obvious that by taking very young leaves, middle aged, and old leaves at the same time, the old leaves are found to respire more rapidly than the middle aged ones, though usually less than the youngest.

The writers are not prepared to say that as protoplasm ages it respires less and that as it gets still older it begins to respire more actively. But it seems that when the amount of CO_2 given off is calculated on the basis of dry weight the rate of respiration increases to some extent after middle age. This is true of the leaves of corn, sorghum, oats, and wheat, but not of sunflower leaves and corn stems.

The amount of CO_2 given off per gm. of dry or of green weight is probably not a good criterion of respiration. That, however, is customary. As a cell increases in size and in age, the total amount of protoplasm probably remains the same while the dry material (cell wall, stored food, etc.) increases. The respiration is presumably connected with the protoplasm. Then if the amount of respiration of an old leaf is calculated on the basis of the total dry weight (cell wall, stored food, etc.) it is obvious that the rate per gm. of dry weight is going to be less than in the young leaves, though the rate per gm. of protoplasm may be the same. The writers have unpublished data to show that as leaves grow older there is an increase in the percentage of dry material up to a certain age, when there is sometimes a decrease. A method which would take into account only the amount of protoplasm would be much more accurate. Perhaps the CO_2 could be calculated on the basis of amino nitrogen or total nitrogen in a plant part.

SUMMARY.

In the present paper it is shown that as the leaves of corn, sorghum, wheat, and oats increase in age there is a decrease in rate of respiration; but that as the leaves become still older (past about middle age) the rate gradually increases.

CONCERNING THE INFLUENCE OF POLARIZED LIGHT ON THE GROWTH OF SEEDLINGS.

By DAVID I. MACHT.

(*From the Pharmacological Research Laboratory, Hynson, Westcott, and Dunning, Baltimore.*)

(Accepted for publication, June 11, 1926.)

The biological effects of polarized light have, until a year or two ago, not been the subject of any scientific experimental study. Recently a few contributions on the subject have appeared. Baly and Semmens published a short note describing the stimulating effect of polarized light on the hydrolysis of starch by diastase (1). These observations were confirmed by the present author (2) who at the same time published several short communications on the pharmacological and other biological effects of polarized light (3) and also described in collaboration with Justina Hill some experiments on the growth of yeast and bacteria (4). In this last communication, Miss Hill and the author called attention to the apparent stimulation of bacterial growth by polarized light. Similar observations on bacteria were published independently by Morrison (5), and recently two Indian investigators have also published a short note claiming that the bacteria of typhoid fever and cholera thrive better in polarized light than in non-polarized light (6). In the present paper the author proposes to describe a series of experiments on the growth of seedlings in polarized and non-polarized light which were begun early in 1924 and continued up to the present time.

Method.

The growth of young seedlings of *Lupinus albus* in a nutrient phytophysiological (Shive's (7)) solution was followed by measuring the elongation of the roots at intervals of 24 hours. The influence of polarized and non-polarized light was studied by means of the following apparatus which was designed jointly by the author and Professor

A. H. Pfund of the Department of Physics of the Johns Hopkins University and was constructed under Professor Pfund's supervision and calibrated by him. A box or cell in the form of a truncated pyramid was constructed 80 cm. high, with a lower base 60 × 45 cm. and upper end about 25 cm. square, the back of the wall of the cell being perpendicular to the base, and the front wall and *door* being slanting. At the upper or small end of the cell a socket is fixed into which is inserted a large round Mazda tungsten nitrogen electric bulb, of 500 watts power, which serves as a source of light. The lower part of the apparatus or cell is divided into two compartments, completely separated from each other by a blackened partition. The light of the Mazda lamp is allowed to penetrate into the chamber on one side of the apparatus after first passing through a dozen plates of smooth glass, placed at the "polarizing" angle, so that this chamber is illuminated with highly polarized light. The light from the same Mazda lamp, on the other hand, is allowed to penetrate into the second or neighboring chamber after first passing through a pile of smooth plates of glass placed *perpendicular* to the line of propagation of the light, so that this second chamber is illuminated with non-polarized light. The number of glass plates in this second pile was adjusted so that the intensity of the non-polarized light was just equal to the intensity of the polarized light in the first chamber. By boring apertures in the floors of the two respective chambers and taking spectrophotographs of the two transmitted lights, it was found that the spectral range of light waves in the polarizing and non-polarizing chambers was the same, the shortest waves transmitted being about 3650 Ångstrom units. The temperatures in the two chambers were nearly the same, not deviating from each other more than 0.5°C.

The source of light was an electric bulb of 700 candle power. This intensity was of course cut down by passage through the piles of plates, but the intensity of the transmitted light in each chamber was made the same by photometric calibration in the Physics Laboratory, performed by Professor A. H. Pfund. The intensities in the two chambers were compared by Professor Pfund by reflecting the lights passing through the two sets of glass plates, from a white surface, and allowing the rays to pass through a Lummer tube. The light from the two chambers was thus reflected *diffusely*, and hence was *depolarized*

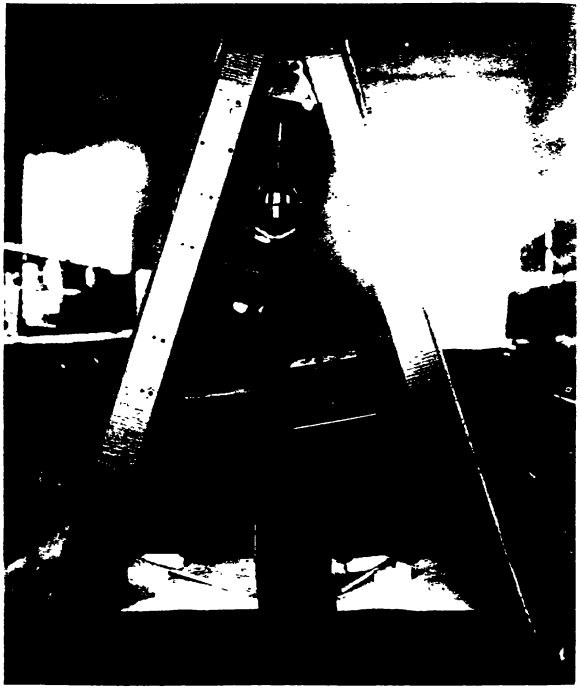

FIG. 1.

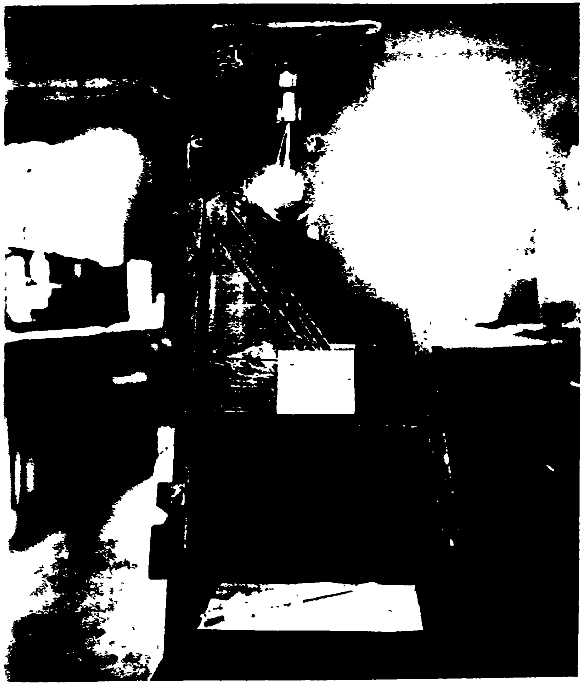

FIG. 2.

FIG. 1. Polarizing apparatus. Front view with door removed.

FIG. 2. Polarizing apparatus. Side view. Right wall removed and showing the chamber illuminated with polarized light.

before the comparison was made. Thus while the eye was used in comparing, it could not be argued that there might be a difference in the physiological effects of polarized and non-polarized lights on the eye. Of course such an objection would be purely hypothetical, as, so far as is known, no difference in the effects on the eye between polarized and non-polarized lights has ever been noted, and if such a difference should be experimentally demonstrated, it would be a fundamental physiological discovery.

In order to make sure that small variations in intensity of the control did not affect the results, a number of experiments were made with the non-polarized light of a slightly greater or slightly lesser intensity than the polarized light (by changing the number of plates in the control chamber). Such variations did not appreciably change the marked effect of the polarized light.[1]

The temperatures in the two chambers were the same to within a fraction of a degree, as indicated by thermometer readings and also by thermographic tracings. Here again a number of experiments were made, in which the temperature in the control chamber was purposely made a little higher or a little lower than in the polarized chamber, respectively, and the results obtained still showed a definite stimulation of growth produced evidently by polarized light.

RESULTS.

Two sets of *Lupinus* seedlings, A and B, generally of 10 each, were carefully measured, then placed in hard glass tubes with Shive's solution, and one set was put in each chamber, Set A in polarized light, and Set B in non-polarized light. The plants were irradiated by the polarized and non-polarized lights, during the daytime, and were left in the dark overnight, when the electric current in the laboratory was turned off. On measuring the growth of the roots of the two sets of plants on the following day it was found that the seedlings exposed to polarized light had grown distinctly more than the other set. The two sets of plants were then interchanged, by placing them in the opposite chambers, that is Set B in polarized light and Set A in non-polarized light, and exposed to the two kinds of light again. On the following day, when the growth of the roots was again measured, it was found that the original "non-polarized" set (B) which was this time exposed to polarized light actually outgrew the original "polarized" set (A) of plants, which, on this second day, was placed in the non-polarizing chamber.

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[1] In other experiments with polarized light, to be published later, intensities were compared by means of a bolometer.

The two sets of plants were again reversed for a second time, and it was found again that the seedlings grew more in polarized light. On reversing the position of the two sets a third time, better growth in polarized light was again observed. Such experiments with *Lupinus* seedlings were made a number of times with the same results. The following protocols will serve as illustrations.

TABLE I.

Set A.					Set B.				
Seedling No.	Normal length	Polarized light.	Non-polarized light.	Polarized light.	Seedling No.	Normal length.	Non-polarized light.	Polarized light.	Non-polarized light
	mm.	*mm.*	*mm*	*mm.*		*mm.*	*mm.*	*mm.*	*mm.*
1	38	49	60	88	1	37	46	57	68
2	35	46	53	80	2	31	50	69	80
3	31	53	60	82	3	23	46	57	64
4	33	39	48	65	4	31	47	62	70
5	24	49	56	71	5	27	43	62	70
6	24	60	64	70	6	34	52	59	64
7	25	50	62	71	7	27	44	70	75
8	29	51	56	64	8	33	47	48	78
9	34	44	56	77	9	33	50	68	77
10	27	41	49	72	10	30	37	65	69
	300	482	564	740		306	462	617	715
		Increment 182.	Increment 82.	Increment 176.			Increment 156.	Increment 155.	Increment 98.

Table I gives the results of an experiment made on 2 sets of plants of 10 seedlings each. Set A was exposed to polarized light on the 1st day, to non-polarized light on the 2nd day, and to polarized light again on the 3rd day. In the case of Set B the order of exposure was reversed, that is on the 1st day the plants were exposed to non-polarized light, on the 2nd day to polarized light, and on the 3rd day again to non-polarized light. The normal or original length of each root is indicated in the first column and the length at the end of each 24 hours in the successive columns. It will be seen that the increment in every case was greater when the seedlings were exposed to polarized light and more than that, when the two sets of seedlings were reversed in respect to the form of radiation, the same phenomenon was noted, namely, the greater growth in polarized light.

TABLE II.*

Set A.						Set B.					
No.	Apr. 26. Original length.	Apr. 27. Polarized.	Apr. 28. Non-polarized	Apr. 29. Polarized.	Apr. 30. Non-polarized	No.	Apr. 26. Original length.	Apr. 27. Non-polarized	Apr. 28. Polarized.	Apr. 29. Non-polarized.	Apr. 30. Polarized.
	mm.	*mm.*	*mm.*	*mm.*	*mm.*		*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1	47	55	69	81	76	1	33	57	70	82	72
2	42	60	78	74	79	2	32	58	61	68	82
3	47	53	65	87	70	3	36	51	68	70	70
4	38	66	69	71	75	4	45	53	75	78	71
5	48	50	62	76	80	5	42	68	74	79	77
6	32	64	64	77	76	6	40	57	71	81	74
7	39	52	65	77	70	7	45	56	71	72	64
8	45	67	62	77	85	8	36	64	71	76	76
9	37	48	69	80	71	9	49	53	64	69	80
10	35	54	68	68	72	10	42	62	76	73	75
11	36	68	66	72	72	11	48	49	61	77	78
12	42	59	68	73	70	12	44	47	60	78	81
13	37	59	74	74	72	13	33	61	73	77	79
14	51	63	63	77	75	14	48	60	69	75	72
15	42	56	58	73	69	15	45	57	63	75	85
	618	874	1000	1237	1112		618	852	1027	1130	1136
		Increment 256.	Increment 382.	Increment 619.	Increment 494.			Increment 234.	Increment 409.	Increment 512.	Increment 518.

In the experiments summarized in Table II two other sets of plants consisting of 15 seedlings each were treated as above, only in this experiment the interchange from polarized to non-polarized light and

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* In a few cases measurement of seedlings shows a shrinkage on the last day.

TABLE III.

Experiment 10.

Series A^1.			Series B^1.		
May 3, 1926.	May 4, 1926.	May 5, 1926.	May 3, 1926.	May 4, 1926.	May 5, 1926.
Normal.	Polarized.	Non-polarized.	Normal.	Non-polarized.	Polarized.
50.1 mm.	67.3 mm.	75.4 mm.	51.9 mm.	64.6 mm.	77.4 mm.
Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.

TABLE IV.

Experiment 11.

Series A^2. (Covered seeds.)			Series B^2. (Covered seeds.)		
May 3, 1926.	May 4, 1926.	May 5, 1926.	May 3, 1926.	May 4, 1926.	May 5, 1926.
Normal.	Polarized.	Polarized.	Normal.	Non-polarized.	Non-polarized.
30.0 mm.	35.7 mm.	39.1 mm.	29.6 mm.	35.3 mm.	37.8 mm.
Mean temp. 22°C.	Seeds covered. Mean temp. 22°C.	Seeds uncovered. Mean temp. 22°C.	Mean temp. 22°C.	Seeds covered. Mean temp. 22°C.	Seeds uncovered. Mean temp. 22°C.

TABLE V.

Experiment 12.

Series A^3. (Covered roots.)			Series B^3. (Covered roots.)		
May 3, 1926.	May 4, 1926.	May 5, 1926.	May 3, 1926.	May 4, 1926.	May 5, 1926.
Normal.	Polarized.	Non-polarized.	Normal.	Non-polarized.	Polarized.
27.2 mm.	36.4 mm.	40.0 mm.	26.4 mm.	34.2 mm.	41.3 mm.
Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.

vice versa was made on 4 successive days until the plants were too long to be measured conveniently. Here again it will be seen that in every case greater growth took place in polarized light. (In this table the order of the seedlings is not the same in each column as they were not placed in individual tubes but in flasks containing several seedlings each.)

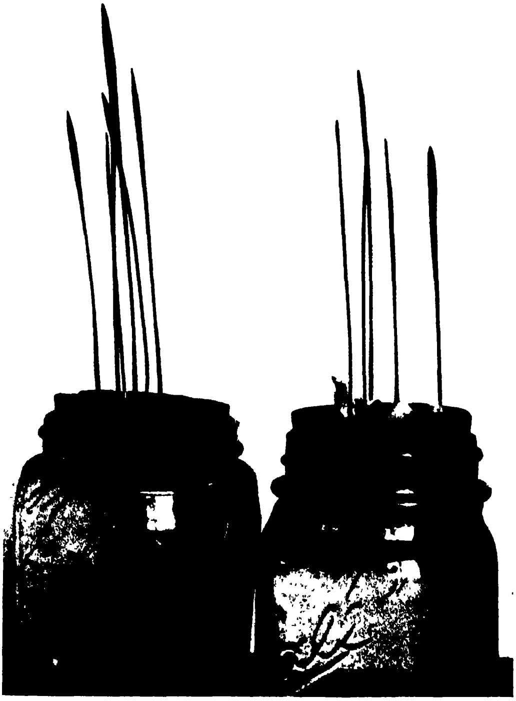
Table III shows an analysis of the above phenomena. In Experiment 10, two sets of plants consisting of 10 seedlings each were placed in polarized and non-polarized light, the figures here given indicating the sum of the root lengths. It will be noted here again that each set of plants grew better in polarized light. Experiment 11 illustrates the effect of wrapping the seeds or cotyledonous portion of the plants with tinfoil. It will be noted that when the beans were wrapped in tinfoil and thus protected from light altogether, growth was exactly the same in both chambers. When, however, these wrappings were removed on May 5th, better growth occurred in polarized light than in non-polarized light. Experiment 12 shows the results obtained

TABLE VI.

Squash Seedlings.

Set A.				Set B.			
No.	Original length	Polarized 24 hrs.	Polarized 72 hrs	No.	Original length.	Non-polarized 24 hrs.	Non-polarized 72 hrs.
	mm.	*mm.*	*mm.*		*mm.*	*mm.*	*mm.*
1	47	58	69	1	45	55	57
2	36	47	54	2	34	37	47
3	32	44	58	3	32	34	47
4	21	27	46	4	22	25	31
5	41	51	71	5	43	54	45
6	31	40	63	6	33	44	80
7	42	47	56	7	37	40	83
8	21	29	52	8	22	35	63
9	19	28	66	9	21	26	34
10	16	26	55	10	18	30	38(?)
	306	397	590		307	380	525
		Increment 91.	Increment 284.			Increment 73.	Increment 218.

when the stems and roots of the seeds were protected from the light but the seed portion was left uncovered. Here better growth took place in polarized light than in non-polarized light thus indicating that the stimulation of growth by polarized light is due to photochemical changes induced in the seed portion. This, of course, harmonized prettily with the findings of Baly and Semmens and the present author in connection with the effect of polarized light on the diastatic hydrolysis of starch.



Polarized. Non-polarized.

FIG. 3. Wheat seedlings.

The author performed most of his experiments on seedlings of *Lupinus albus*. A number of experiments, however, were also performed on other plants. In Table VI are the results obtained with squash seedlings. Here again it will be noted that better growth occurred in polarized light than in non-polarized light. A number of experiments were made with wheat seedlings; as these seedlings grow several roots which are difficult to measure, the growth was studied by measuring the elongation of the stems and leaves. In Table VII and Fig. 3 are shown the results obtained in one such experiment. Two sets of 6 seedlings each were exposed to polarized light and non-polarized light and the length from the root to the tip of the blade of each seedling was measured. It will be noted that better growth took place in polarized light. In several other experiments with wheat seedlings the growth of the plants was studied by weighing them. In this way it was also found that the seedlings exposed to polarized light weighed distinctly more than those exposed to non-polarized light. In Table VIII and Fig. 4 is shown the effect of polarized light on two seedlings of the sunflower, the length of the stems was measured and it will be noted better growth took place in the polarizing chamber.

TABLE VII.

Wheat Seedlings.

(See Fig. 3.)

Set A.	Set B.
In polarized light.	In non-polarized light.
6 seedlings.	6 seedlings.
Original length from roots to tip of blade, 402 mm. 3 days later, 656 mm. 4 " " 921 "	Original length from roots to tip of blade, 406 mm. 3 days later, 588 mm. 4 " " 804 "

TABLE VIII.

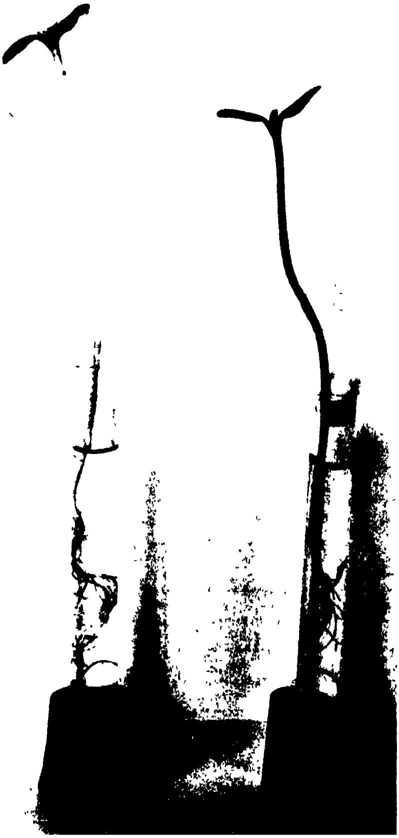
Helianthus (Sunflower).

(See Fig. 4.)

Seedling A.	Seedling B.
Dec. 16, 1925. Length of stem, 120 mm. Placed in polarized light.	Dec. 16, 1925. Length of stem, 120 mm. Placed in non-polarized light.
Dec. 18, 1925. Length of stem, 140 mm.	Dec. 18, 1925. Length of stem, 125 mm.
" 19, " " " " 147 "	" 19, " " " " 127 "

SUMMARY.

While these experiments are not exhaustive, a sufficient number have been made to warrant the statement that the effect of polarized light of the visible spectrum on the growth of various seedlings and more particularly on the growth of *Lupinus albus* is somewhat different from that of non-polarized light. This is especially convincing in view of the results obtained with double sets of plants which were alternately exposed to polarized and non-polarized lights of the same



Polarized. Non-polarized.

FIG. 4. Sunflower seedlings.

intensities and at the same temperature. In every experiment thus performed the set which was placed in a polarizing chamber grew better. It is, furthermore, interesting to note that the phenomenon above observed did not take place when the seed portion of the plants was protected from light by wrapping with tinfoil. This agrees well with previous findings concerning the action of diastase on starch in polarized light. The above researches will be continued on a more elaborate scale but the results so far obtained are deemed worthy of publication in the form of a preliminary communication at the present time.

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ON CURVES OF GROWTH, ESPECIALLY IN RELATION TO TEMPERATURE.

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I.

Growth generally manifests an accelerated velocity during the mid-portion of a developmental cycle, so that the curve of bulk with time is sigmoid. This fact gave rise (Ostwald, 1902; Errera, 1899–1900; Robertson, 1907–08) to the view that the accumulation of material during growth may be described by the equation for a first-order process in which one of the products of the transformation acts as catalyst. This conception has had a vigorous enlargement (Robertson, 1923), looking toward the description of the velocity of development as governed by a succession of first-order chemical processes, "master reactions," which are self-accelerated.

Several difficulties are connected with the application of this idea. One of these is that in fitting simple logistic (autocatalytic) curves to actual data, it has usually been assumed (*cf.* Robertson, 1923; 1925–26) that the curve of growth in any cycle is symmetrical about a mid-point of inflection. This follows from the nature of the equation commonly taken to give the course of such an autocatalyzed process:

$$\frac{dx}{dt} = K\,x\,(A - x), \tag{1}$$

where A signifies the initial endowment of the growth-promoting precursor, x the amount formed after time t. The point of inflection in the integral curve of this equation is located at

$$x = A/2.$$

A method of testing the sufficiency of this form of the autocatalysis equation is afforded by the study of rates of development as controlled by temperature. The autocatakinetic relationship (Lotka, 1925) is of so very general a character, arising in any kind of a situation where a limited progress is facilitated by the conditions created through its initiation[1] but progressively inhibited by proportionately enforced exhaustion, that some test of this sort is necessary if the form of growth curves is to be satisfactorily understood. Thus if the simple logistic were an adequate description of growth velocity, temporary alteration of the temperature of the development would not be expected to modify the temperature characteristic (Crozier, 1924–25, *a*) for the remainder of the development. Since the temperature characteristic for the velocity constant K must be constant, changing the temperature therefore merely multiplies the time coordinates of the growth curve by a constant. The temperature characteristics obtained from what may be termed "partial developmental periods" should be the same as for the total developmental interval (within one cycle). There is adequate indication (Bliss, 1925–26) that this may not be the case. Consideration of this fact results in a modification of the formulation of the "autocatalytic" curve of growth. The modification has the merit of greater consonance with chemical theory, and of indicating a direct interpretation of the sort of results to be expected when the temperature is changed during the course of a developmental cycle. It may also give some light upon the nature of temperature characteristics for development at constant temperatures.

Let it be supposed that at the beginning of a developmental cycle there is available an unrenewable quantity, A, of a substance giving rise to another, x, which determines the velocity of growth. We are especially interested in "velocities of growth" as measured by the reciprocals of the times required to attain a given stage of development. We will suppose that the material A gives rise to x by a first order reaction, and that x serves as catalyst for this change. The reaction $A \rightarrow x$ will therefore be governed by a velocity constant (K_1) proper to it in the absence of the influence of x, and also by the velocity constant due to catalysis by x. The decomposition of A must therefore be conceived as made up of two parallel reactions, and its differential equation is then

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[1] An interesting instance is given by the growth of knowledge of the variety of the amino acids, as plotted by Cohn (1925). A still different type of S-shaped curve may result from estimations of growth in which the numbers of cells of colonial protozoans are counted (Fauré-Fremiet, 1922); in certain species the mode of dichotomy results in the curve for number of individuals against time appearing "autocatalytic."

$$\frac{dx}{dt} = (K_1 + K_2\,x)\,(A - x), \tag{2}$$

where K_2 is the velocity constant associated with x as catalyst.

The velocity of formation of x will pass through a maximum when

$$x = \frac{K_2\,A - K_1}{2\,K_2}. \tag{3}$$

Therefore, if any change of condition, such as temperature, influences K_2 and K_1 unequally, the form of the curve connecting x with time will be changed and the point of inflection will move to a new relative position. Thus when K_2 is made relatively smaller, the inflection point occurs earlier (assuming A the same), and the shape of the curve is significantly altered.[2]

It is to be noticed that in such a system the point of inflection is found at $x = A/2$ when K_1 is of inappreciable magnitude; whereas, in case K_2 is very small the equation approaches that of the usual monomolecular curve without detectable autocatalysis.

II.

Integration of (2) yields

$$t = \frac{1}{K_1 + K_2\,A}\,ln\,\frac{A\,(K_2\,x + K_1)}{K_1\,(A - x)} \tag{3}$$

The curve of this equation may be applied to various series of observations; for example, to the growth in height of sunflower plants (data of Reed and Holland, 1919). The fit (Fig. 1) may be made distinctly better than when the "simple" curve of autocatalysis is employed. An adequate test of the descriptive validity of this equation for growth might be sought in the curve of increasing weight for a mammal, where temperature variation may be neglected. Donaldson's data on the growth of the rat (Donaldson, 1915) are suitable for such a test, though undoubtedly complicated by the cyclic character of growth in mammals (Donaldson, 1906; Robertson, 1907–08; Brody and Ragsdale, 1922–23). Robertson (1923) has fitted to this data two "fused" logistic cycles, but the agreement is not especially good. In Fig. 2 it is shown that these data are fitted sufficiently well by the autocatalytic equation in the form $dx/dt = (K_1 + K_2 x)(A - x)$, except that for a

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[2] This formulation seems simpler, and leads to more suggestive consequences so far as concerns the planning of experiments, than does Robertson's (1923) suggestion of the pseudo-reversible character of the "master process" in growth; moreover, it would appear to be the obviously correct equation for an autocatalytic system. Robertson pointed out that if a reverse process in the growth equation is of higher order than the direct, the curve of increasing bulk with time becomes asymmetric about the inflection point; many if not most growth-cycles are in fact asymmetric (*cf.* Brody, 1925–26). A different mode of dealing with the asymmetry has more recently been employed by Robertson (1925–26), which amounts to assuming that x has a positive value at the beginning of a cycle.

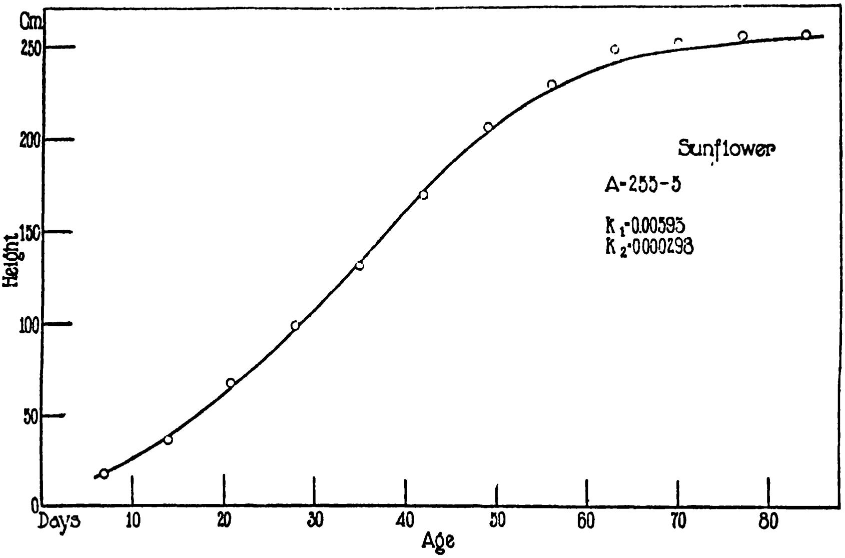

FIG. 1. Growth in height of the sunflower; data of Reed and Holland (1919); the curve is that of the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A\,(k_2 x + k_1)}{k_1\,(A - x)},$$

the cycle starting at $x = 5$ cm., with $k_1 = 0.00595$, $k_2 = 0.000298$; A, the mature height, is taken $= 250$.

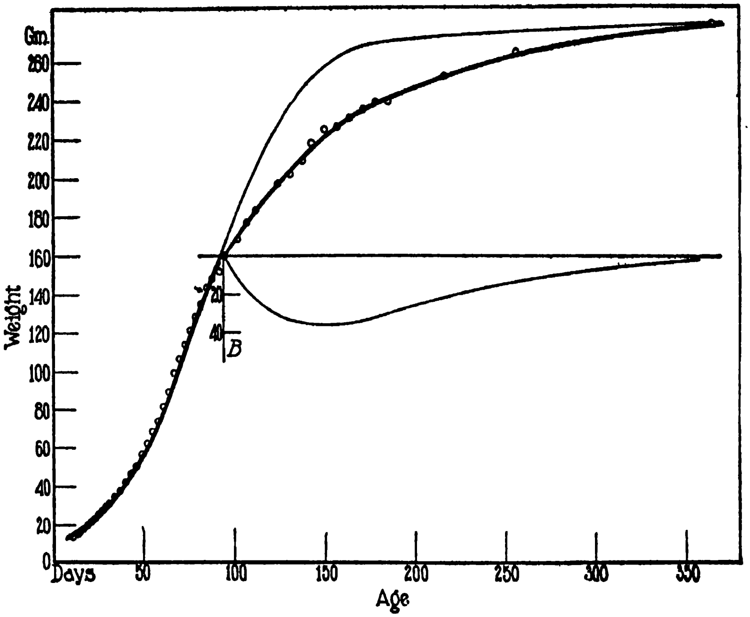

FIG. 2. Data on the growth of unmated male white rats (Donaldson, 1
there fairly well to the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A\,(k_2 x + k_1)}{k_1\,(A - x)}$$

for the first 100 days. It is assumed that the weight reflects the amount of a growth-determining material x, produced in a reaction $A \rightarrow x$, which is catalyzed by x; that the value of A is given by the maximum weight (for convenience, 280 gm.); and that the cycle starts at $x = 10$ gm. The constants for the curve as drawn are $k_1 = 0.00135$, $k_2 = 0.000135$; x = weight -10.

Beyond 95 days age, it may be supposed that the material x is inhibited by another, B, produced in a system of the type $M \underset{K_3}{\rightarrow} B \underset{K_4}{\rightarrow} N$, where M is unrenewable. The thin inverted curve is the curve of this process with $M_0 = 121$, $k_3 = 0.01663$, $k_4 = 0.02375$; the equation is

$$B = M_0 \frac{k_3}{k_4 - k_3} \left(e^{-k_3 t'} - e^{-k_4 t'}\right),$$

t' being counted from $t = 95$.

The curve drawn through the observed weights (circles) is obtained by subtracting B from x.

period after 100 days age the deviation is great. It is an interesting fact, which may perhaps be used to obtain another view of the mechanism of growth "cycles," that the deviation after 100 days from the formula fitting the earlier course of the data and also its terminal range, may be accounted for quantitatively and exactly by assuming that beyond age 95 days the growth-controlling substance, x, is inhibited (but not destroyed) in proportion to the amount of a substance B produced in a system of the type $A \xrightarrow[K_3]{} B \xrightarrow[K_4]{} C$. Assuming suitable values of A, K_3, and K_4 as found from the deviations of the data from the calculated autocatalytic curve, the accessory curve in Fig. 2 gives the values of B to which the deficiency of x is assumed to correspond. The curve drawn through the plotted points is obtained by taking the difference between B and the calculated x. From this standpoint it may be legitimate to regard the decrease in growth velocity between two cyclic accelerations as due to the presence of an inhibitor, which disappears with time and does not destroy x. It should be possible to relate an inhibition of bulk-increase to known physiological events in the organism dealt with. For the rat it can be pointed out that the maximum in the "correction curve" (B) occurs at an age of 150 days, at which time (Donaldson, 1924) the activity of the thyroid seems to attain a definite maximum and then to decline. This implies that thyroid activity is to be taken as hindering growth in bulk (at this age). Miss King's measurements of growth of rats (Donaldson, 1924) may be fitted in a similar way, with the maximum of the "correcting curve" at very nearly the same age. There is indication that the peculiar growth curve of man (summary of data in Davenport, 1926) may be accounted for in a similar way, with the maximum growth inhibition (male) at about 14 years; but the deviation from calculated autocatalytic curves is so extensive as to make difficult the adjustment of a "correction curve."

III.

The way in which the curve of equation (3) may be modified by changing the temperature is illustrated in Figs. 3 and 4. We may assume that for the completion of a developmental stage, say an instar or other definite interval in the differentiation of an insect, there must occur the production of a definite amount of a substance,

x, arising in a first order reaction $A \rightarrow x$, and that x is a catalyst for the reaction. We must suppose that in general the temperature characteristic for the catalytic effect of x will not be the same as that for the catalytic influence promoting the reaction apart from the action of x. As already pointed out, this will so affect the shape of the curve that the graphs at two temperatures are not superimposable. If one-half of the developmental period under consideration were to be passed at t°C., this would not mean that one-half of the development would be completed, since the curve is not symmetrical. Then on passing to

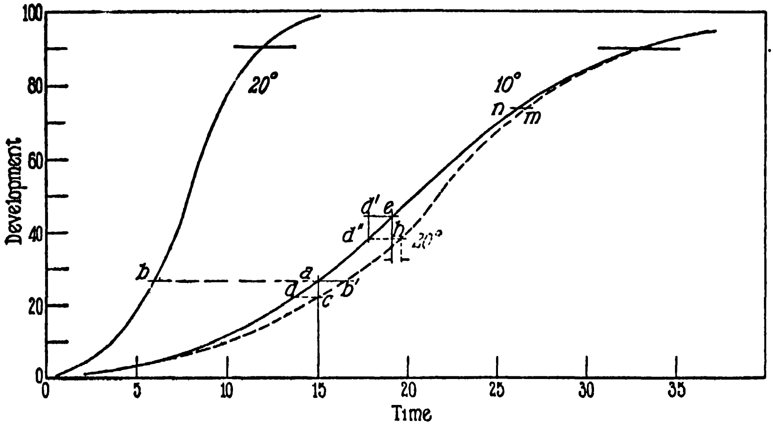

FIG. 3. Curves of the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A(k_2 x + k_1)}{k_1 (A - x)},$$

with $A = 100$ in each case. (Time is in arbitrary units.)

At 20°, k_1 is assumed $= 0.010$

k_2 " " $= 0.005$

At 10°, k_1 " " $= 0.005$ (*i.e.*, $Q_{10} = 2$)

k_2 " " $= 0.0017$ (*i.e.*, $Q_{10} = 2.94$)

These curves are not superimposable; the dashed curve is that for the 20° conditions, expanded by multiplication of the abscissæ by a factor which makes the curves coincident at $x = 0.90A$.

If such curves are assumed to underlie development, and if we assume that at completion of a given stage x = a definite fraction of A, say = 90 per cent, then Q_{10} for velocity of development is (from the curves) $32.92 \div 12.02 = 2.74$. As A is assumed larger and larger, the Q_{10} ratio for velocity of development approaches nearer and nearer to the Q_{10} for k_2. (If the assignment of Q_{10}'s is reversed, Q_{10} for $x = 0.90A$ is 2.13.) Additional details are discussed in the text.

another temperature, the development still to be completed would be (depending on the alteration in the ratio K_1/K_2) either more or less than the fraction uncompleted before the transfer; and the time required to produce the necessary fixed amount of x would be correspondingly greater or smaller than what would be calculated; hence the apparent temperature coefficient for the velocity of the partial development would not be the same as for the total development when passed at constant temperatures. (No account is here taken of differences produced by "breaks" in the curve of developmental rate with temperature.)

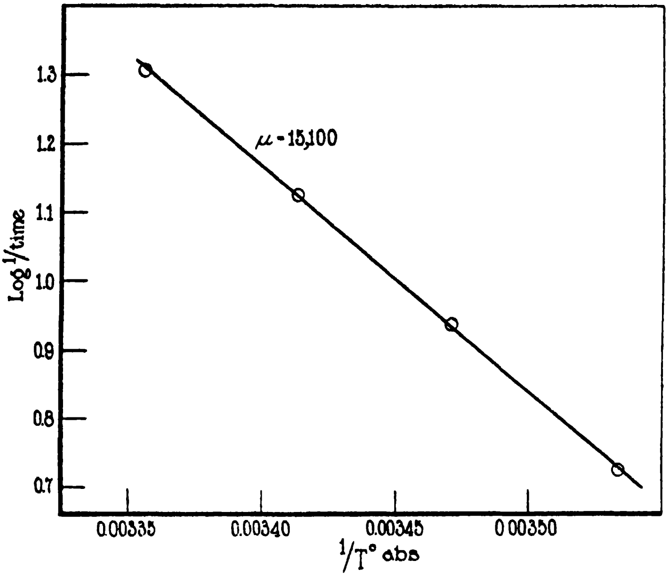

FIG. 4. From the autocatalytic curve, with assumed values $A = 500$, $k_1 = 0.08$, $k_2 = 0.0015$, and with μ for $k_1 = 11{,}000$, for $k_2 = 16{,}000$, values of the time are calculated to give $x = 490$. The reciprocals of these times are plotted logarithmically against $1/T°$ abs., at 10°, 15°, 20°, 25°C. The relationship is almost exactly rectilinear, and the temperature characteristic obtained from the graph (fitted line) is $\mu = 15{,}100$. This shows that in a system of the sort discussed in the text it is possible to estimate an apparent critical increment which agrees fairly closely with that for one of two catalytic velocity constants concerned in an autocatalytic activity.

The assumption of a definite amount of x as marking the termination of a developmental interval is required because the end-product of growth is the same at each temperature. It is convenient to regard this amount as a fraction of *A*, the original endowment. With arthropods in particular, the succession of developmental intervals is so definite that one is forced to think of their termination as controlled by "trigger effects," such as might be provided through the accumulation of a governing substance to a critical concentration rather than by the asymptotic approach to completion which would correspond to the extinction of *A* in our model.

It is desirable to show that deviations in apparent temperature coefficient when part of a developmental period is passed at another temperature, such as those recorded by Bliss (1925–26), may be systematically accounted for. (1) If, in Fig. 3, the organism is maintained at 10° for a calculated fraction of the developmental time interval (*i.e.* to *a*) it is then ahead of schedule by comparison with the curve of development at 20° (*b′*); hence the supposed portion of the development still to be completed (from *b′*) is actually less, on transfer to 20°, and the apparent temperature coefficient in consequence is larger than if rates of complete development at 10° and at 20° are compared.

(2) On the other hand, if exposure to low temperature occurs during an intermediate fraction (from *c* to *e*), the development is "behind schedule" at *c* (by the amount *c a* corresponding to the time *c d*), and on arrival at time *e*, calculated to give a certain fraction of the total developmental time, this will actually be fallen short of so far as concerns differentiation by the amount *h*, so that the degree of development is then in fact that represented at point *d′*. If now the organism be brought back to 20°, the developmental level is indicated at point *h*, which is ahead of that assumed at time *e;* during subsequent development at 20° the developmental course still to be completed is thus less than that calculated and hence appears faster; but with the conditions shown the apparent acceleration is less than in the first case, where transfer from the 10° to the 20° curve results in a considerable time-saving and therefore in a marked increase of calculated temperature coefficient.

(3) Again, if transfer from the 20° curve to the 10° be made late in development, as at point *m*, time is lost, because the relative develop-

ment is greater at the same time along the 10° curve; therefore the calculated temperature coefficient is actually less than that obtained from uninterrupted developments at 10° and at 20°.

For the prepupal period of *Drosophila*, with a normal temperature characteristic 16,850, Bliss (1925–26) found that in experiments of type (1) the apparent μ was 20,220; of type (2), 18,770; of type (3), 16,570. There is additional indication of just this sort of relationship in data given by Titschak (1926) for the rate of development in the clothes moth *Tineola*. The particular curves drawn in Fig. 3 are of course intended merely to show that it is possible to explain such results. It is significant that this explanation turns upon properties of an equation which describes with some precision the time course of growth and differentiation such as can be visibly evidenced by increasing weight, and which it is therefore legitimate to suppose may describe even more accurately the progress of developmental changes measurable only by the incidence of their end-results.

IV.

Although few investigations of growth permit very precise estimations of critical thermal increments, it is nevertheless important that there is indication of diverse magnitudes of μ, the temperature characteristic, for different cases; and that these values are suggestively close to those known to be associated with various other vital processes (Crozier, 1925–26). A careful investigation of a particular developmental stage in *Drosophila* has been made by Bliss (1925–26); Brown (1926–27) has determined the temperature characteristics for a developmental interval in cladocerans; the values of μ derived for these phenomena correspond to values repeatedly found in processes of quite different sorts (Crozier, 1925–26; Crozier and Stier, 1925–26). This is a striking fact, because one would be inclined to believe that chemical mechanisms controlling growth might be very different from those having to do, for example, with the regulation of the heartbeat or of breathing movements. This suspected difficulty might be overcome if it could be shown that the velocity of development, or the duration of life in particular stages, is determined, not by the magnitude of some simple underlying chemical change, but by the velocity with which this change is taking place. Northrop (1925–26) has indeed shown that the duration of life in *Drosophila* does not depend upon the transformation of a definite amount of energy (Rubner), since the amount of CO_2 produced during life is not a measure of the life duration.

Aside from this possibility, however, the curve of equation (3) has some interesting properties which indicate another mode of interpretation. The reciprocal of the time for production of, say, $x = 0.90\,A$, will have a temperature coefficient which depends on the magnitudes of K_1, K_2, and A, and of the temperature coefficients of K_1 and K_2. If A be put $= 100$, and $K_1 = 0.010$, $K_2 = 0.005$, at 20°, with the respective temperature coefficients Q (10°–20°) $= 2.0$ and 2.94, then Q (10°–20°) for $1/t$ will be 2.74. Thus the temperature coefficient for $1/t$ may agree very closely with that for one of the two velocity constants.

This kind of relationship may be illustrated by one particular set of assumptions as to the values of A, K_1, and K_2, when it is supposed that $\mu = 11{,}300$ for K_1 and $\mu = 16{,}500$ for K_2. We desire to see whether the values of log $1/t$ will in such a case give a rectilinear relationship to the reciprocal of the absolute temperature, as seems to be the case in growth. It is seen that with very small deviation, such as would probably be overlooked in practical cases, they, indeed, do give this relationship (Fig. 4).[3] This is sufficient to demonstrate that an autocatalytic system in which two velocity constants are implicated may permit the approximate evaluation of temperature characteristics, and that these may even be quite close to those corresponding to the action of specific catalysts. The calculated curve is not exactly rectilinear, but in practice it may be very difficult to decide the origin of deviations at the extremes of the temperature range. It may be pointed out, however, that one may, from this standpoint, expect to find evidence of: (1) slight differences in critical increments when contrasting growth phenomena with other common activities, (2) relationships between log $1/time$ and $1/T^\circ$ abs. which are not exactly rectilinear, but concave toward the $1/T^\circ$ axis, and (3) deviations of critical increment when development is passed in part at one temperature, in part at another. The testing of these, especially (1) and (2), is at present handicapped by absence of precise data.

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[3] It is easily seen that with other values of K_1, K_2, the agreement could be very much closer. The values used in this illustration are chosen merely to show the slightly curvilinear character of the plot (Fig. 4), which would otherwise be detected with difficulty.

It may be pointed out that calculations of 1/ *time for completion* of a stage of development based upon the integral form of $dx/dt = (K_1 + K_2\,x)\,(A - x)$ do not yield "breaks" in the curve of log (1/ *time*) *vs.* 1/ $T°$ *abs.*, even when such temperature characteristics are assumed as reverse the sign of the difference between K_1 and K_2 at the extremes of temperature. In such a case the μ calculated from the times required to produce $x = 0.90\,A$ agrees rather closely with that μ, whether of K_1 or K_2, which happens to be smaller. It is of interest that in a system of this kind it is the magnitude of the *temperature characteristic*, rather than of the velocity constant, which chiefly determines the apparent μ of the resultant.

V.

In discussing growth rates as controlled by temperature it must be kept in mind that the momentary growth rate may vary with time. This may result not merely from the form of the growth-curve, but also because the growth-curve may smooth out recurrent cyclic variations in rate. An instance has already been given (Crozier, 1924–25, *b*), derived from Leitch's measurements of root elongation in the sweet pea. The temperature characteristics in this case differ markedly, depending upon the time period (0.5 hour or 24 hours) involved in the measurement. This is probably related to the fact that cell division occurs at a fairly definite point in a diurnal growth-rhythm (Stålfelt, 1921). Other kinds of complexity are not unexpected, for no one in his senses regards growth as an uncomplicated process. Lehenbauer's (1914) data show that the mean hourly elongation of maize seedlings increases abruptly at 20°, and with time. The average μ above 20° is about 15,500. Where such "breaks" occur in the temperature graph the effects to be expected when time is a significant variable are difficult to predict.

It is, nevertheless, of interest to examine the available measurements of growth to see if they yield anything in the nature of consistent temperature characteristics. A number of observers have studied the growth of fungal colonies as related to temperature. The data are not always presented in the most directly usable form, nor can the probable retarding effects of changes in the medium be discounted with any sureness. In the growth of such colonies on agar plates it is to be supposed that in the absence of retarding or accelerating effects the rate of enlargement should be constant. A correct measure of growth, assuming only extension in area to occur, would be given by the increase in area per unit of circumference per unit time. When growth of such a colony gives a sigmoid curve with time it is certainly inappropriate to regard the curve as describing an inner autocatalyzed growth-controlling process, and hence probably useless to attempt analysis of its precise relations to temperature. Fawcett (1921) records the growth in diameter of colonies of several fungi. These figures have been used to calculate the areal increase per unit time

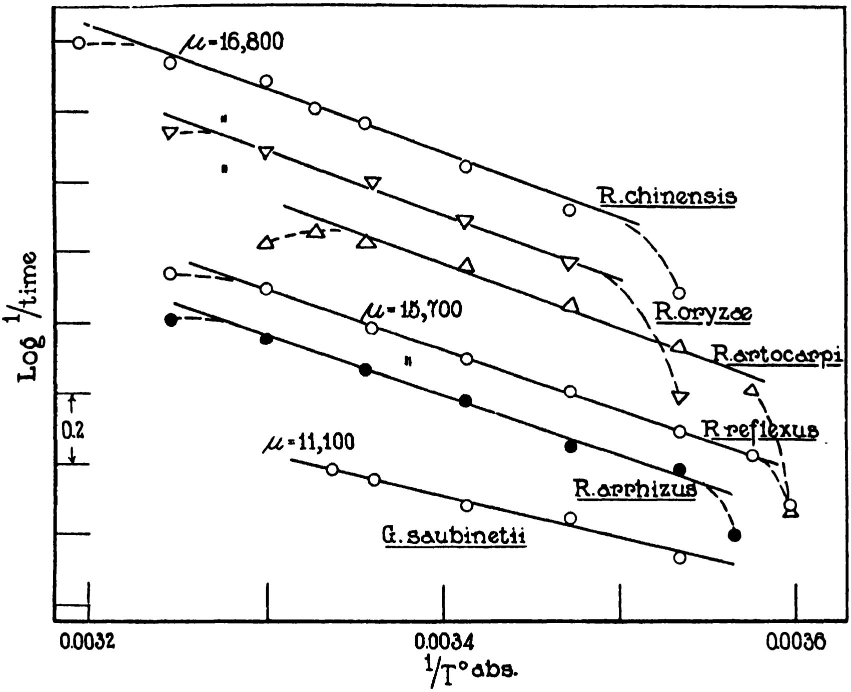

FIG. 5. The velocity of early growth in 5 species of *Rhizopus*, measured by the time required for the germinating hypha to become as long as the diameter of the spore (data from Weimer and Harter, 1923); and the growth of *Gibberella saubinetii*, taken as 1 ÷ time for colony to attain a diameter of 2.5 cm. (by interpolation from data of MacInnes and Fogelman, 1923). It is apparent that aside from terminal deviations such as are usually encountered the temperature characteristics closely resemble those calculated from data on respiration (Crozier, 1924–25, *b*).

(24 hours) per unit of growing edge at successive intervals. The rate of enlargement so computed changes with time, in such a way as to suggest that more extensive measurements would, at each temperature, pass through a maximum. The initial growth of the colony so affects the medium that growth is accelerated. Estimations of the temperature characteristic of the inner growth-promoting process would, therefore, require the separation of the effects of temperature upon growth from those involving changes induced in the medium.

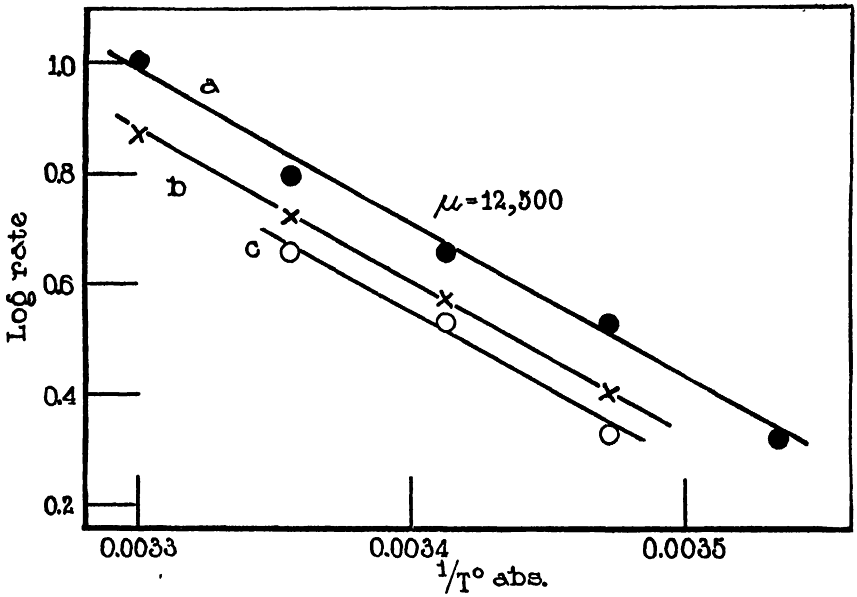

FIG. 6. The rate of decay of sweet potatoes inoculated with *Rhizopus*. The temperature characteristic is sensibly independent of the end-point chosen (*i.e.*, the amount of destruction, presumably determined in greater part by the activity of the fungus). Data from Lauritzen and Harter (1925).

Weimer and Harter (1923) studied the germination and growth of a number of species of *Rhizopus*. From their curves it is possible to plot the reciprocals of the times required for the germ tubes to grow until the length equals the diameter of the spores; these figures provide mean velocities of growth for the attainment of a constant amount of growth (Fig. 5). The agreement with the Arrhenius formula is usually excellent, since the deviations at high and at low temperatures are no different from those generally encountered in other vital systems. The values of μ accord sufficiently well with those known in other growth phenomena.

In such a case it is to be presumed that the temperature characteristic obtained is not a property of the constants in an autocatalytic system, but pertains merely to a metabolic mechanism immediately responsible for increase in bulk.

MacInnes and Fogelman (1923) measured the growth of colonies of *Gibberella saubinetii*, and from their figures it is possible to obtain by interpolation the time required at several temperatures to form a colony of given size (diameter). Data from Lauritzen and Harter (1925) may be used in a similar way, giving the time required for *Rhizopus* to produce a given amount of decay in sweet potato. These measurements are plotted in Figs. 5 and 6, where the corresponding temperature characteristics are indicated.

These instances illustrate the applicability of the Arrhenius equation but they do not permit critical examination of the meaning of the corresponding temperature characteristics. It can be said merely, that in general the critical increments resemble in their magnitudes those already encountered in various other vital activities. A similar vagueness of interpretation is necessary in connection with studies of growth and regeneration in animals. Moreover, and aside from experimental difficulties in temperature control, many cases are disturbed by the fact that the developmental process under examination was not exactly begun at the temperature indicated, but the lot of organisms was distributed to thermostats some little time *after* development had begun. Bearing this difficulty in mind, we may, however, examine the velocity of development in several instances. It should be noted that there is direct justification for regarding a morphological end-point as a legitimate end-point for our purpose, provided it be timed with precision; Terroine and his associates (Terroine, Bonnet, and Joessel, 1924; Barthélemy and Bonnet, 1924) have indicated that thermal acceleration of development to a constant stage (*e.g.* to absorption of external gills, in the tadpole; germination of seeds) does not modify the utilization of energy during the growth process,—in other words, the chemical "balance" is the same at the same stage of development. But in many instances it may be impracticable to obtain a significant morphological end-point.

The speed of early cleavage of the fertilized ovum has been measured by Loeb and Wasteneys (1911) and by Loeb and Chamberlain (1915), for the egg of *Arbacia*. These two series of measurements are concordant, and exhibit critical increments 12,400; 21,000; 41,000,

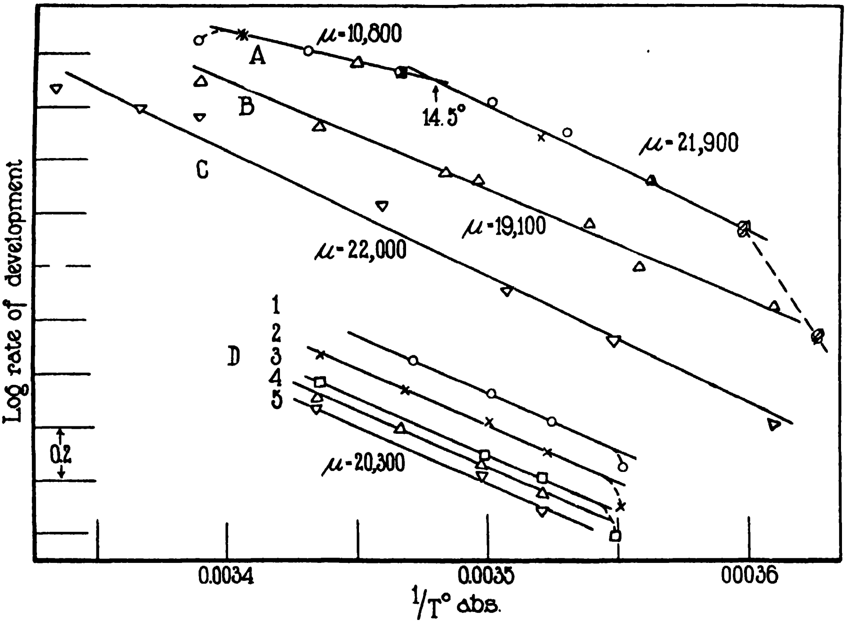

Fig. 7. Data upon the rate of cleavage and upon early stages of development in anurans.

A. Processes involved in the first cleavage, *Rana* (from Krogh, 1914).

B. From first cleavage to disappearance of yolk-plug (from Lillie and Knowlton, 1897).

C. From first, second, or third cleavage to disappearance of yolk-plug (Lillie and Knowlton, 1897).

D. From fertilization to: (1) medullary groove, (2) external gills, (3) 3 gill plumes, (4) a length of 7.0 mm., (5) a length of 7.8 mm.; *Rana* (Data from Krogh, 1914).

It is clear that there is a measure of consistency in the occurrence of $\mu = 20{,}000 \pm$. This is indicated also in some fragmentary data from Barthélemy and Bonnet (1924). Hertwig's data (*cf.* Cohen, 1901) also show parallelism in the effect of temperature at different stages of development, but indicate for the intermediate range of temperatures a distinctly lower μ, 17,000±. Estimations of the rate of growth of the body, and of the tail, in *Rana* and *Bufo*, agree in indicating $\mu = 24{,}000 \pm$ (data from Lillie and Knowlton, 1897).

with "breaks" at 11° and at 20° (Crozier, 1924-25, *b*). The velocity of segmentation in eggs of the frog (first cleavage) is plotted in Fig. 7, from data by Krogh (1914). The velocity of subsequent early

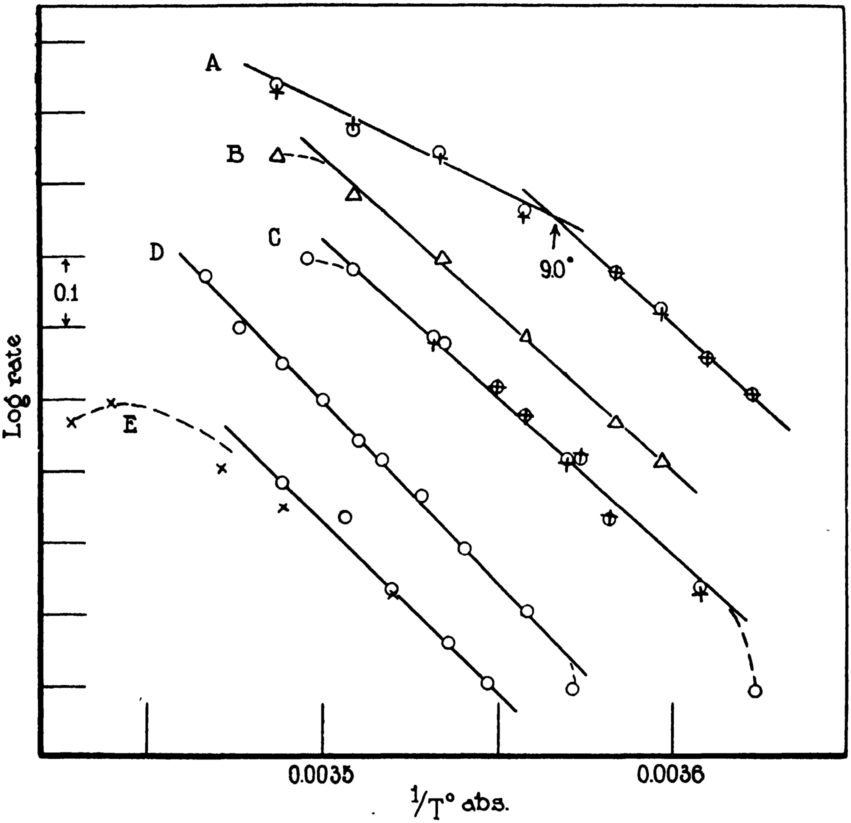

FIG. 8. Data upon the development of teleosts:

A, *Gadus morrhua* and *G. æglefinus*; *B*, *G. merlangus*; average velocities of development up to hatching; observations by Dannevig (in Johansen and Krogh, 1914); for *A*, μ = 11,800 and μ = 20,200, with break at 9.0°C; for *B*, μ = 20,000.

C, Plaice; to length 4.6 mm., circles; to length 4.9 mm., crosses; the two series brought together by multiplying the members of the second by a factor; μ = 20,000. (Johansen and Krogh, 1914).

D, *Hypomesus olidus* (Higurashi and Tauti, 1925); μ = 23,700.

E, *Plecoglossus altivelis*; two series, one from Higurashi and Tauti (1925), the second from Higurashi and Nakai (1926); μ = 23,000.

It should be noted that in the cases of deviation at extreme temperatures there is independent evidence of abnormal differentiation.

development of the frog, in Krogh's experiments, exhibits a constant increment not significantly different from that for the mid-range of temperatures (15° to 4.7°) in the case of the first cleavage, but the critical temperatures are different.

It is noteworthy that in the case of amphibian development the temperature characteristics calculated from the data of Lillie and Knowlton (1897) upon the early development of *Rana* and of *Amblystoma* (Fig. 7) may be said to agree quantitatively with the magnitude obtained over the lower temperature range (4° to 15°) in Krogh's (1914) experiments.

The rate of development of teleost embryos has been studied by Krogh and others. The more extensive series of observations are collected in Fig. 8. More recent experiments on the rate of insect development provide data showing for *Dytiscus marginalis* $\mu = 19{,}300$, for *D. semisulcatus* $\mu = 20{,}000$ (to 15°) and $\mu = 10{,}400$ (data from Blunk, 1923). Some figures from Ziegelmayer (1926) for rate of development of *Cyclops* give $\mu = 15{,}700$. Such data have been treated in a different way by Krogh (Johansen and Krogh, 1914), but the most direct utilization of the measurements shows that there is a striking constancy in the occurrence of $\mu = 20{,}000$, with one series showing $\mu = 24{,}000$; thus there is evidenced a suggestive parallelism with the values obtained for amphibian development.

For the full interpretation of such graphs it is necessary to know the effect of the preliminary period during which the objects (*e.g.*, fertilized eggs) have been maintained at some constant or nearly constant temperature before distribution to thermostats at different temperatures. In the light of the view previously set forth in this paper it would be expected that if at all prolonged this preliminary interval might well have a definite and detectable effect. It would be expected to accentuate any innate departure from the rectilinear character of the plots, or might seriously affect the apparent magnitude of the temperature characteristic. This especially makes it impossible to use critically much of the early material on insect development (*cf.* Sanderson, 1910; Sanderson and Peairs, 1913), although it supplies interesting suggestions. In the most carefully conducted experiments the adherence to rectilinearity is certainly satisfactory (*cf.*Crozier, 1924–25, *b*; Bliss, 1925–26; Brown, 1926–27).

The fact that the same temperature characteristic holds for mean growth velocities at different points on a curve of development (Fig. 7) must be taken to signify that within the range considered the shape of the underlying curve very nearly is the same at different temperatures; hence, that only one "velocity constant" is materially effective, or else that if more than one be involved, their temperature characteristics are the same.[4]

The net result may be stated by saying that while the control of growth velocities by chemical reaction velocities seems adequately shown, it is yet highly desirable that further data be secured by improved methods; there is some indication that the controlling reactions may belong in categories with those found by their temperature characteristics to be implicated in other and quite diverse vital processes. There is as yet scarcely sufficient evidence to verify the prediction that the curve relating log velocity of growth to $1/T°$, when "velocity" = reciprocal of time required to reach a defined stage, should be slightly curvilinear. But there is indication that growth velocities, where evidenced as constant rates of increase, adhere satisfactorily to the Arrhenius formula; and even when we may quite reasonably expect that an "autocatalytic" system is involved, the agreement is often as good as might be desired. The values of the temperature characteristics secured for growth phenomena are quite varied, yet they cluster rather definitely about the following magnitudes; 7–8,000; 11–12,000; 16–17,000; 20,000; 24,000; 27,000 (this summary is based upon studies of a number of cases additional to those specifically mentioned in this article). Consideration of the properties of the equation which describes the velocity of an autocatalytic process (Section IV) shows how this sort of result may be obtainable.

[4] With regard to velocities of regenerative growth, which in certain cases at least appear to adhere to typical growth curves, there does not exist any considerable body of data. We may cite the following instances. The regeneration of hydranth in *Tubularia* (Moore, 1910) has been cited in an earlier paper (Crozier, 1924–25, *b*). Measurements of the rate of regeneration (morphyllaxis) in planarians indicate a high temperature coefficient (Lillie and Knowlton, 1897; Vandel, 1921–22), but are insufficient for analysis.

VI.

SUMMARY.

The velocity of growth, taken as the reciprocal of the time required to attain a given size or stage of development, obeys with some exactness the Arrhenius equation for relation to temperature. The values of μ, and the type of "breaks" found in the curves connecting velocity and temperature, are similar to those found in the case of various other vital activities. More precise data, particularly from experiments in which parts of the given developmental stadium are passed at different temperatures, may strengthen present indications that this relationship is not absolute. It is pointed out that the equation for an autocatalytic process, taken as descriptive for growth, predicts particular sorts of deviation under these conditions, which have in one instance been obtained experimentally; and may at the same time nevertheless permit the apparent temperature characteristic for (average) growth velocity to agree rather closely with that for one of the two velocity constants present in the correct autocatalytic equation.

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EXIT OF DYE FROM LIVING CELLS OF NITELLA AT DIFFERENT pH VALUES.*

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(Accepted for publication, July 6, 1926.)

I.

INTRODUCTION.

The purpose of the present paper is to outline a theory[1] of the penetration of a dye (brilliant cresyl blue) into living cells of *Nitella*,[2] and to examine how far this theory is in harmony with the facts found in studying the exit of the dye from the cell.

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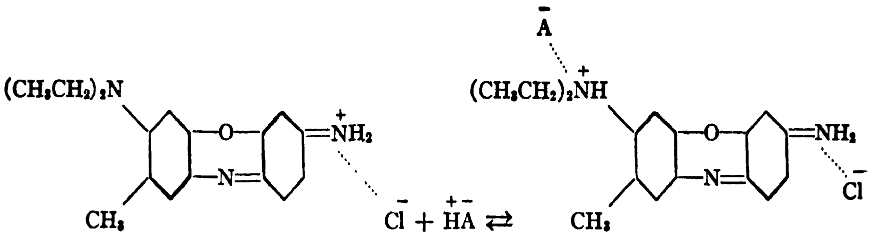
* This work was in part done when the writer held a Fellowship in the Biological Sciences of the National Research Council, Washington, D. C.

[1] Another theory, previously proposed by the writer (Irwin, M., *J. Gen. Physiol.*, 1922-23, v, 727), regards the rate of penetration and the final equilibrium as dependent primarily on the concentration of the salts of proteins or weak acids, XA, at the surface of the cells, which combine with DS, to form a compound capable of diffusing into the sap. But on further experimentation the writer has concluded that there are many objections to this theory. The most serious objection of all is found in the fact that the rate of penetration seems to be directly proportional to the ratio $\frac{\text{DB}}{\text{DS}}$ for each particular dye at various pH values, when a comparison of the relative rates at these pH values is made by the writer among several basic dyes having different apparent dissociation constants.

Penetration is regarded by the following writers as dependent on the combining of dye ions with proteins: Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, lx, 354. Mathews, A., *Am. J. Physiol.*, 1898, i, 445.

[2] *Nitella* is a fresh water plant with multinucleate cells up to 4 inches in length, having an outer cell wall, beneath which is a very thin layer of protoplasm surrounding a relatively large central vacuole. The pH value of the sap in the vacuole is about 5.6, and the sap contains about 0.1 M halides in addition to organic acids and protein.

The theory [3,4] states that the dye exists in (at least) two forms, one of which is the "free base"[5] which we may call DB, soluble in chloroform, and another, which we may call DS, little or not at all soluble in chloroform. When the pH value of the solution increases a part of DS is changed to DB (and *vice versa*), so that at each pH value these two forms are in equilibrium with each other (and possibly with a third form, which may be a pseudo base found at still higher pH values). The nature of DB is still an open question. According to the theory of Hantzsch and others[6] both DB and DS may be treated as salts, DB being a quaternary ammonium salt which is capable of undergoing a further salt formation owing to the presence of other basic groups. The following[7] may make this clear by using cresyl blue as an example.



DB: colored blue in water and red in chloroform. Soluble in chloroform and water.	decreasing pH ⟶ increasing pH ⟵	DS: colored blue in water. Soluble in water but little or not at all in chloroform.

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[3] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

[4] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561.

[5] In former papers (see Foot-notes 3 and 4) this free base was called DOH for convenience but in order to avoid any possibility of confusing DOH with the dye hydrate (which may not be the form we are dealing with), it will be called DB hereafter.

[6] For a discussion of the theory of indicators see Henrich, F., Theories of organic chemistry, translated by John Johnston and Dorothy Hahn, London, 1922.

[7] For the formula see Conn, H. G., Biological stains, Geneva, New York, 1925, 51.

In the case of brilliant cresyl blue, DB and DS have the same color. If the above description of DB and DS is correct DB may be a strongly dissociated salt like DS. On the other hand, DB may be regarded as an undissociated molecule, and DS a strongly dissociated salt. Experiments are being carried out by the writer to determine the behavior of DB in this respect. Dr. Grinnell Jones has kindly determined the change in the conductivity of chloroform with and without the dye. When 100 cc. of pure chloroform were shaken up with 1 liter of M/150 borate buffer solution at pH 9, the specific conductivity of this chloroform was found to be 6×10^{-10}. When the same volume of chloroform was shaken up with 1 liter of M/150 borate buffer solution at pH 9 containing 3.5×10^{-4} M brilliant cresyl blue until there was practically no dye left in the aqueous solution, the specific conductivity of this chloroform was found to be 233×10^{-10} (about forty times greater than that of the chloroform containing no dye). This indicates that some or all of the dye exists in the chloroform in dissociated form.

The behavior of these two forms is very different. Apparently[8] DB can pass through the cell rapidly but DS penetrates extremely slowly or not at all.

Although it is evident that the form of the dye[9] which principally penetrates the cell is the one which is soluble in chloroform, the writer does not wish to subscribe without reserve to the lipoid theory in its present form.

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[8] In connection with this, it may be assumed that DS corresponds with the ions and DB with the undissociated molecules, of a weak base, acid, or salt. In the paper by Hoagland and Davis (Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, vi, 47) it is stated that the time of exposure of the living cells of *Nitella* to solutions containing NO_3 or Br ions, is a matter of days, before a detectable amount is found in the sap even at a favorable external pH value, temperature, and condition of light. The time of exposure, on the other hand, in the case of the penetration of cresyl blue into *Nitella* at a favorable external pH value, and temperature, is a matter of seconds. This fact agrees very well with Osterhout's suggestion (see Foot-note 10) that the undissociated molecules enter the cell, while the ions enter only very slowly or not at all. Furthermore, it agrees with the writer's theory, since the halides are only very slightly soluble in substances like chloroform and benzene, and in this respect the halides correspond with DS of the basic dye.

[9] The following writers state that some basic dyes enter the living cells as a free base: Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. It is stated by Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360, that an acid dye, 2,6,dibromophenol indophenol penetrates *Valonia* only in the form of an undissociated molecule.

Previous experiments[1,3] have shown that when the external concentration is kept constant throughout the experiment, the entrance of the dye is found to follow the equation:

$$\frac{dx}{dt} = k\,(a - x)$$

where a = the concentration of the dye in the sap at equilibrium and x = the concentration of the dye in the sap at the time t, while k = the velocity constant. When the values of x are calculated from this equation they are found to agree very closely with the observed.

This agreement of course does not determine whether the process is governed by diffusion or by chemical reaction.

The temperature coefficient, furthermore, for the rate of penetration between 20°C. and 25°C. is very high (above 4) but this again may not necessarily indicate that the process is controlled by a chemical reaction rather than by diffusion.

Until further knowledge is obtained concerning the temperature coefficient for the diffusion of substances through an artificial system which more or less closely resembles the living cell of *Nitella*, and in which the passage of solute molecules or ions from one solvent phase to another probably does not depend upon forces of the sort usually regarded as "physical" it is not possible to determine whether the rate is governed by simple diffusion or by chemical reaction.

It is quite possible that under some circumstances it is controlled by diffusion and under other circumstances by chemical combination.

Since we are unable at present to decide whether the rate is controlled by diffusion or by chemical reaction let us for the sake of simplicity assume that it is diffusion, since in this case the mechanism is less complicated, and proceed to analyze the data on this basis. After this is done we shall discuss the alternative hypothesis; *i.e.*, that the rate is controlled by chemical reaction.

If we assume that the rate is controlled by diffusion, the mechanism may be explained as indicated by Diagram A (the cell wall being omitted). In this diagram nothing is said regarding combination of the dye with a cell constituent but this does not indicate that there is no possibility of such a reaction in the protoplasm. It is regarded, for the present, as not affecting the rate; it is therefore omitted in order to simplify the diagram.

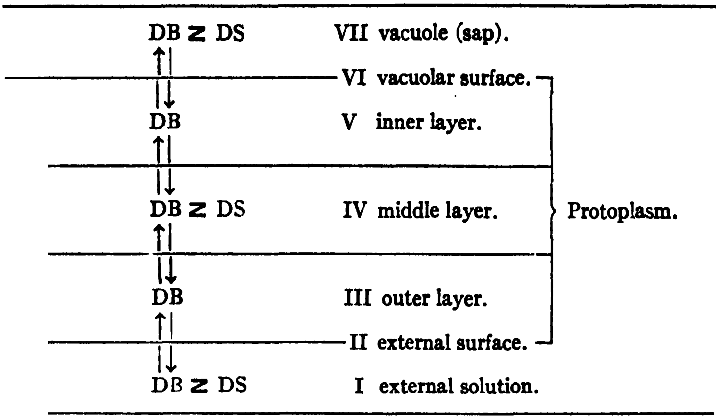

Diagram A. The cell wall is omitted for convenience.

Diffusion is designated by the sign ⇄; equilibrium between DB and DS by the sign ⇌. For convenience the process is divided into seven parts. The inner and outer layers (III and V) are hypothetical. The vacuolar surface (VI) represents the protoplasmic surface in immediate contact with the sap, while the external surface (II) represents the protoplasmic surface in contact with the external solution. In I, IV, and VII, DB is in equilibrium with DS and a constant ratio of $\frac{DB}{DS}$ is maintained in each medium as long as the conditions remain unchanged. The concentration of DB in one part is in definite relation with that of DB in any other part of the diagram. Thus, for example, if the concentration of DB in the external solution (I) is changed, successive changes in the concentration of DB in all the parts of the cell take place. It is assumed that DB diffuses through III and V while DS diffuses to such a slight extent as to be negligible in the present case. For penetration the velocity of diffusion of the dye, DB, from I to VII is greater than that from VII to I while for the exit the velocity from VII to I is greater. An equilibrium is established when the velocity of the inward diffusion is equal to the outward diffusion. At equilibrium the concentration of DB in the vacuole is proportional to the concentration of DB in the external solution depending on the apparent[10] dissociation constant and on the distribution coefficient $C = \frac{\text{DB in the sap}}{\text{DB in the external solution}}$. If C is 1, the concentration of DB in the sap at equilibrium is equal to the concentration of DB in the external solution. If C is lower than 1, the concentration of DB in the sap will be lower than that of DB in the external solution, and *vice versa.* Since there is maintained in the sap a definite ratio of $\frac{\text{DB}}{\text{DS}}$, the concentration of DS depends on the concentration of DB. Thus the final concentration of the total dye (DB and DS) in the sap at equilibrium will depend on the apparent dissociation constant of the dye $\left(\textit{i.e.}\text{ the ratio of } \frac{\text{DB}}{\text{DS}}\right)$ in the sap, on the partition coefficient of DB, and on the ratio of $\frac{\text{DB}}{\text{DS}}$ in the external solution.

According to this scheme it is possible to study the mechanism either of the penetration of the dye into or of the exit from the vacuole, by determining the concentrations of the dye (DB plus DS) in the sap, as long as the color of DB does not differ from that of DS. The rate of penetration will increase and that of exit will decrease when the concentration of DB just outside the external surface (II) is increased. The reverse is the case when the concentration of DB in the sap is increased as, for example, by any change in the medium which changes the apparent dissociation constant of the dye $\left(\textit{i.e.}\text{ the ratio of } \frac{\text{DB}}{\text{DS}}\right)$, or by a change in the solubility of DB in the sap.

The theory thus outlined accords with the facts previously obtained for the penetration of dye.[3,4] Let us now consider whether it accords with the facts observed in connection with the exit of dye from the cell. We shall proceed upon the assumption that when a stained cell is placed in a solution containing no dye, the dye comes out according to the scheme outlined in Diagram A (the outward process VII I from the sap to the external solution). We shall test this assumption by experiments.

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[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

II.

Methods.

Living cells of *Nitella* were placed in 8.6×10^{-6} M brilliant cresyl blue at pH 8.2 for 17 minutes, when the concentration of the dye in the sap reached 7.94×10^{-5} M. (The concentration of dye in the sap was determined colorimetrically as described below.) The cells were then removed from the dye solution, gently wiped with a damp cloth, and distributed in solutions at different pH values (pH 5.4 to 8.2) containing no dye. At definite intervals a few cells were removed, and the concentration of the dye in the sap was determined by the colorimetric method as follows: The end of each cell was cut and the sap was gently squeezed out onto a glass slide; the sap was drawn up into a capillary tube the color of which was matched with that of the capillary tube of the same diameter containing a standard dye solution.

In order to avoid experimental error from the presence of the dye in the external solution, only six cells were placed in 200 cc. of solution (without dye) and the solution was constantly stirred and changed every 15 seconds. This method gives the maximum velocity constant for each experiment, *i.e.* there is no further increase in the velocity of the exit of the dye if the frequency of stirring and of changing the solutions is increased.

The concentration of the external dye solution (8.6×10^{-6} M) is chosen because this is sufficiently dilute to avoid error due to the adhering of the dye to the surface of the cell, after the cell is removed and wiped with a damp cloth. The cellulose wall is not stained when cells are placed in this concentration of dye at pH 8.2.

If too high an external dye concentration is used, the exit of the dye from the sap is hindered (even when the cell wall is not stained) when the cells are removed from the dye solution and placed in a solution without dye, though the latter solution is constantly stirred and changed. This decrease in the rate of the exit of the dye is due in all probability to the fact that the dye adhering to the surface of the cell cannot be washed away quickly enough. This complication may be eliminated by using an external solution which is at least seven times more dilute than the concentration of the dye in the sap which is chosen for the experiments. In order to be absolutely certain that the concentration used avoids this experimental error the experiment was repeated with still lower concentrations but it was found that the result was not altered.

All possible care was taken to have all the cells used at one time as alike as possible, so that the differences in the rates were due chiefly to the experimental conditions and not due to the difference in the condition of the cells before the experiments began. Unless otherwise stated, the *Nitella* used was obtained from Cambridge and the experiments were carried out in early fall when the cells were in excellent condition.

The tests for early stages of injury are very unsatisfactory. The appearance of masses of chlorophyll in the expressed sap, the rapid exit of halides from the intact cell, and the loss of turgidity all indicate advanced stages of injury rather than the first. For this reason it is desirable to control the experiments in some way so that we have a more or less uniform method of detecting the condition of the cell immediately after the experiments. To do this, after each experiment, some of the cells were tested for injury by placing them in distilled water, and for 4 days[11] at intervals of every few hours the percentage mortality was compared with that of the control cells (fresh cells placed in distilled water under same conditions). It was found that the percentage mortality of the cells thus treated was about the same as that of the control cells.

These experiments, like those heretofore described[3,4] by the writer, were carried out in an incubator at 25 ± 0.5°C., into which diffused light was permitted to enter through small ventilating holes.

The buffer solutions used were M/150 phosphate mixtures. The pH values of these buffer solutions were determined by means of the hydrogen electrode. The dye used was that of Grübler, and was taken from the same stock bottle as the one used in the writer's experiments[3,4] on penetration.

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[11] It is not desirable to continue such a test for any longer period since the comparison between the test cells and the control cells becomes more doubtful, in view of the fact that even the control cells do not live indefinitely in the laboratory.

III.

Analysis of the Time Curves.

That lowering of the pH value of the external solution (containing no dye) hastens the exit of the dye from the sap of living cells of *Nitella* is indicated[12] by the curve in Fig. 1. At low pH values (5.4 to 6) the process may be followed until practically all the dye has come out of the sap without causing injury to the cells, but at higher pH values injury or death may occur. The curves given in Fig. 1 represent the process when the cells are not injured.

At higher pH values of the external solutions here employed it is probable that all the dye in the sap would eventually be found to come out of the vacuole if we could continue the experiment long enough and still keep the cell from being injured. The analysis of the time curves therefore is made on the assumption that at the end of the process the concentration of the dye in the sap is zero at all external pH values.

The velocity of diffusion is assumed to be proportional to the difference between the concentration of DB in the sap and that of DB in the external solution. According to the present theory, there is a definite ratio of $\frac{\text{DB}}{\text{DS}}$ in the sap and in the external solution, so that for mathematical treatment the concentration of DB in both may be replaced, for convenience, by the concentration of the total dye (DB and DS) which we actually measure. Since the concentration of the dye in the external solution is approximately zero, we may in the following equation let a denote the initial concentration of the dye in the sap, x the concentration of the dye that has disappeared from the sap at time t, and k the velocity constant of diffusion. We may then write:

$$\frac{dx}{dt} = k\,(a - x) \quad \text{or} \quad k = \frac{1}{t} \log \frac{a}{a - x}$$

When k is calculated for each time curve it is found to decrease

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[12] These results confirm those obtained previously by the writer (Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223). It may be added here that the writer has chosen to study the exit of the dye by the method presented in this paper first, because other methods offer greater complications.

TABLE I.

Exit of Brilliant Cresyl Blue from Living Cells of Nitella at Varying External pH Values at 25°C.

The process is represented by the equation $\frac{dx}{dt} = k\,(a - x)$, where a is the initial concentration of dye in the sap, $a - x$ is the concentration of dye in the sap at the time t, and k is the velocity constant. The calculation is made with a 20 inch slide rule. $a = 7.94 \times 10^{-5}$ M for all external pH values.

t	pH 5.4			pH 5.7			pH 6.0			pH 6.8			pH 7.5			pH 7.8			pH 8.2		
	$a - x$ obs.	k	$a - x$ calc.	$a - x$ obs.	k	$a - x$ calc.	$a - x$ obs.	k	$a - x$ calc.	$a - x$ obs.	k	$a - x$ calc.	$a - x$ obs.	k	$a - x$ calc.	$a - x$ obs.	k	$a - x$ calc.	$a - x$ obs.	k	$a - x$ calc.
min.	M × 10^5		M × 10^5	M × 10^5		M × 10^5	M × 10^5		M × 10^5	M × 10^5		M × 10^5	M × 10^5		M × 10^5	M × 10^5		M × 10^5	M × 10^5		M × 10^5
1	4.83	0.215	4.96	4.83	0.215	5.07	5.59	0.152	5.62	6.21	0.107	6.30									
2	3.05	0.208	3.11	3.28	0.192	3.23	4.00	0.148	3.96	4.83	0.107	5.01	6.14	0.055	6.02	6.56	0.041	6.46	6.9	0.030	7.08
3	2.15	0.189	1.94	2.31	0.179	2.06	2.76	0.153	2.81	4.14	0.094	3.98	5.00	0.067	5.24	5.87	0.043	5.82	6.80	0.023	6.68
5							1.40	0.151	1.40	2.76	0.092	2.51	3.80	0.064	3.97						
7													3.28	0.055	3.02	3.80	0.046	3.84	5.52	0.023	5.31
10																2.59	0.049	2.82	4.49	0.025	4.47
Average.....		0.204			0.195			0.151			0.100			0.060			0.045			0.025	

slightly as the concentration of the dye (DB and DS) in the sap approaches zero (Table I). This decrease in the value of *k* may be explained on the ground that there is a relative increase in the velocity of the inward process due to an increase in the ratio of DB in the film just outside the external surface (II) to the DB in the outer layer (III) and hence to the DB or to the total dye (DB and DS) in the sap, since toward the end of the process, where there is a very little dye (DB and DS) left in the sap, the amount of DB in the film just outside the external surface (II) may no longer be a constant fraction of DB in the outer layer (III) and of the total dye in the sap as was the case at the beginning of the process (this will be discussed later on). Since this decrease in the con-

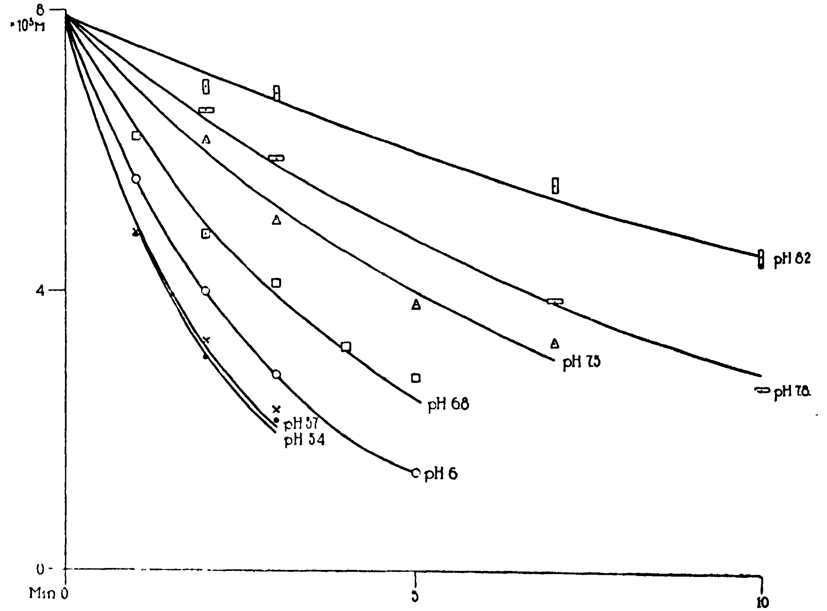

FIG. 1. Time curves showing the exit of brilliant cresyl blue from the living cells of *Nitella* at different external pH values at 25°C., when the initial concentration of the dye in the sap is 7.94 × 10^{-5} M. The ordinates represent the concentrations of dye in the sap, while the abscissæ represent time. The curves as drawn represent the calculated values of the concentration of the dye in the sap, while the symbols represent the observed values. Each point on every curve is an average of fifty experiments, and the probable error of the mean is less than 8 per cent of the mean.

stants is not very great, the average is taken of all the velocity constants at each external pH value. When the values of $a - x$ are calculated for each time curve by using the average value of k, thus obtained, they are found to agree fairly closely with the observed except toward the end of the process, where there is an indication that the calculated values are slightly lower than the observed, as shown under pH 5.4 and 5.7, Table I.

In connection with the analysis of the time curves it may be well to repeat the following in order to avoid misunderstanding. (1) It makes no difference in the form of the time curve whether we measure DB alone or DB + DS in the sap, since DB and DS stand in constant relation as long as the conditions, such as the pH value of the sap, remain unchanged. We actually measure DB plus DS (called the "total dye" for convenience) in the sap and the analysis of the time curves is made by using the concentrations of the total dye. (2) The concentration of the total dye in the sap is affected by the concentration of DB in the other parts of the cell, and in the solution outside the cell. Thus, for example, if the concentration of DB in the outer layer (III in Diagram A) is decreased, the concentration of DB and hence that of the total dye in the sap is decreased.

When the temperature coefficient between 20° and 25°C., for the exit of the dye at pH 5.7 and also at pH 7.8 was determined, Q_{10} was found to be about 4.

IV.

The Relation of the Velocity Constant to the pH Value of the External Solution.

The time curve for each external pH value is found (see Table I) to follow approximately the equation:

$$\frac{dx}{dt} = k\,(a - x)$$

where a denotes the initial concentration of DB in the vacuole minus the concentration of DB in the external solution (which in this case is practically zero), x the amount of DB that has diffused out of the vacuole at the time t, and k the velocity constant. In both cases, DB for convenience is put equal to the total dye which is actually measured.

If the stirring and frequent changing of the external solution kept the concentration of DB equal to zero just outside the external surface of the protoplasm (which is designated by II, the external surface, in Diagram A), we should expect to find the same values of *k* for all external pH values. But the value of *k* decreases with an increase in the external pH value, as shown in Fig. 2, and the explanation for this may be that the concentration of DB at the external surface changes as the external pH value changes. Let us assume that just outside the external surface of the protoplasm there is a liquid film more or less protected against the direct effect of stirring by the cellulose wall and that in this film a certain amount of DB (a certain percentage of which is at once transformed to DS) collects as it comes out of the cell; also that the total amount of DB

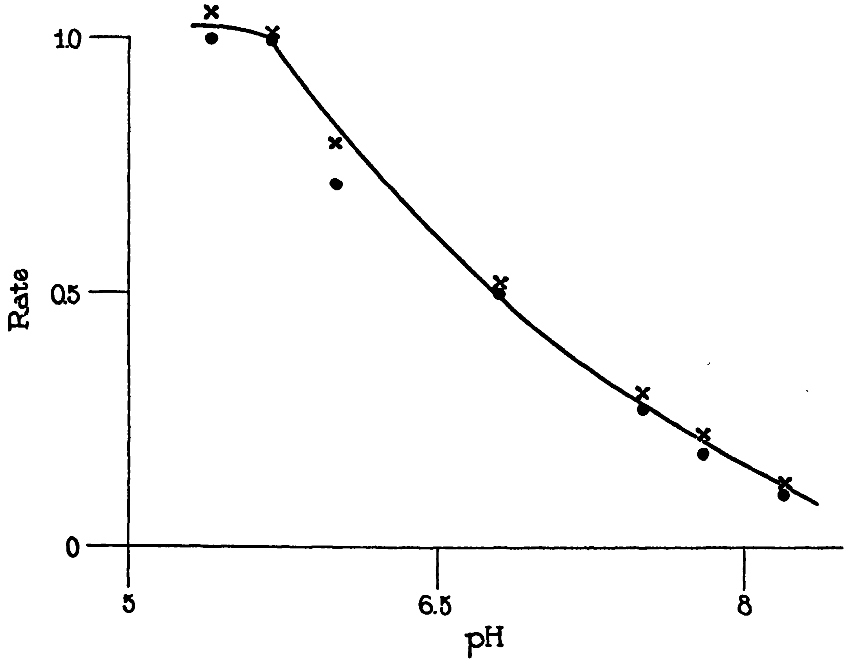

FIG. 2. Curve showing the relation of the external pH values to the rates of the exit of the dye (symbol ×), and also to the velocity constants (symbol ●). The ordinates represent the rates, and also the velocity constants multiplied by 5.2 (for convenience of plotting), and the abscissæ represent the pH values of the external solutions.

which comes out is approximately the same in all solutions containing no dye, but the per cent of it which remains in the form of DB depends on the pH value of this film (which is assumed to be approximately the same as that of the external solution since the latter can penetrate freely through the cellulose wall into the film) since a certain amount of DB will change to DS depending on the pH value of the film.

When the cell is removed from the dye, wiped, and placed in a solution in which no dye is present, DB begins to diffuse from the vacuole, the protoplasm, and the film just outside the protoplasmic surface. We may assume that the concentrations in all of these places fall off together, so that when the concentration in the vacuole has fallen to half the value it had at the start, that of the protoplasm and the film will also have fallen to approximately half value. In that case we may regard the falling off in the protoplasm and in the film as following an approximately unimolecular curve (since we have found this to be true of the dye in the vacuole) and consequently the amount of DB in the film will be an approximately constant fraction of that in the sap. If we call the dye in the sap $a - x$ and designate as y the amount of DB in the very thin layer in immediate contact with the outer surface of the protoplasm we may write:

$$y = b\,(a - x)$$

in which b is a constant expressing the amount of DB in the film as a fraction of the amount of DB (which for convenience is put equal to the total dye) in the vacuole throughout the process at any one external pH value.

When the pH value of the external solution changes the value of b will also change, since the per cent of DB in the film will be altered. In order to see how this will affect the rate of exit of the dye from the vacuole, let us first consider the case where there is no effect of y on the velocity constant. Since according to our analysis of the time curves, the dye comes out of the vacuole in a unimolecular fashion, we may write

$$\frac{dx}{dt} = k_1\,(a - x)$$

in which k_1 is the velocity constant of the process when dye is present on one side of the surface only. This expression gives us the rate of exit of the dye when there is no dye in the film. When dye is present in the film a certain amount diffuses back into the cell. The true rate[13]

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[13] Criticism may be made as to this method of mathematical treatment since it involves the consideration of the diffusion of DB through only one very thin surface, when in fact the protoplasm of *Nitella* consists of more than one such layer. Even if we were to treat the entire protoplasmic layer as one surface, the question may be raised as to how far we are justified in considering the protoplasm to be thin enough for such a mathematical treatment. If we consider the diffusion of DB through two surfaces, the vacuolar and the external surfaces (II and VI in Diagram A), one at a time, then we may modify the analysis given in the text in the following manner. The amount diffusing inward through the external surface in unit time when DB is present in the film only $= k_1y$ (just as described in the text). Let us assume that the amount diffusing inward in unit time through the vacuolar surface when there is no DB in the vacuole is a constant fraction of k_1y so that we may put this amount equal to ck_1y, in which c is a constant. The amount diffusing outward in unit time through the vacuolar surface when DB is present in the vacuole but not in the protoplasm or in the film (DB fictitiously introduced into the vacuole without getting into the protoplasm) is $k_1\ (a - x)$. Hence we take the difference between the amount going outward through the vacuolar surface and the amount passing inward through the vacuolar surface and we have:

$$\frac{dx}{dt} = k_1\,(a - x) - k_1cy$$

put $y = b\,(a - x)$ in which b is a constant (just as given in the text) then

$$\frac{dx}{dt} = k_1\,(a - x) - k_1bc\,(a - x)$$

$$\frac{dx}{dt} = (k_1 - k_1bc)\,(a - x)$$

or on integration

$$k_1 - k_1bc = \frac{1}{t}\log\frac{a}{a - x}$$

$$bc = \left(k_1 - \frac{1}{t}\log\frac{a}{a - x}\right) + k_1$$

Since we are not able to verify the values of the constants b and c experimentally, and since assuming a value for either b or c is very unsatisfactory, we are not able to explain the mechanism any more convincingly than we have done in the text.

Experiments are now in progress to see whether it is possible to determine the of exit is the resultant of these two processes and may be found by subtracting the amount which would diffuse inward if dye were present on one side only from the amount that would diffuse outward if dye were present on the other side only. Hence we may write

$$\frac{dx}{dt} = k_1 (a - x) - k_1 y$$

Substituting in this equation the value $y = b\,(a - x)$ we have

$$\frac{dx}{dt} = k_1 (a - x) - k_1 b\,(a - x)$$

$$\frac{dx}{dt} = (k_1 - k_1 b)\,(a - x)$$

or, on integration,

$$k_1 - k_1 b = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 (1 - b) = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 = \left(\frac{1}{t} \log \frac{a}{a - x}\right) \div (1 - b)$$

and

$$b = \left(k_1 - \frac{1}{t} \log \frac{a}{a - x}\right) \div k_1$$

We may put $k_1\ (1 - b) = k$; substituting the value $k = \frac{1}{t} \log \frac{a}{a - x}$ we have $b = \frac{k_1 - k}{k_1}$.

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constants experimentally in order that we may know in greater detail what the controlling factor is for the rate of penetration into and that of exit of the dye from the vacuole.

It might be possible that the rate of penetration into and that of the exit of the dye from the vacuole are controlled by the rate of diffusion of DB through only one very thin layer in the cell, (the layer through which the diffusion of DB is the slowest). Whether this is represented by the external surface (II in Diagram A) or by the vacuolar surface (VI) or by some other part of the cell, we are not able to state definitely at present. In all probability under varying conditions the controlling layer varies.

The values of a, x, and t may be obtained experimentally but the values of k_1 and b cannot be obtained in this way. We may, however, assume an approximate value of k_1 and we are justified in doing this since we are interested in relative rather than in absolute values. The analysis of the time curves shows decreasing values of k (see Table I and Section III) with increasing external pH values. When such values of k are plotted as ordinates and the concentration of DB in the external solution expressed as per cent of the total dye, (obtained from the experiments[4] made by the writer on the distribution of the dye between chloroform and water) are plotted as abscissæ, we obtain the curve shown in Fig. 3. The curve indicates that when DB (and consequently y) equals zero, the value of k is somewhat above

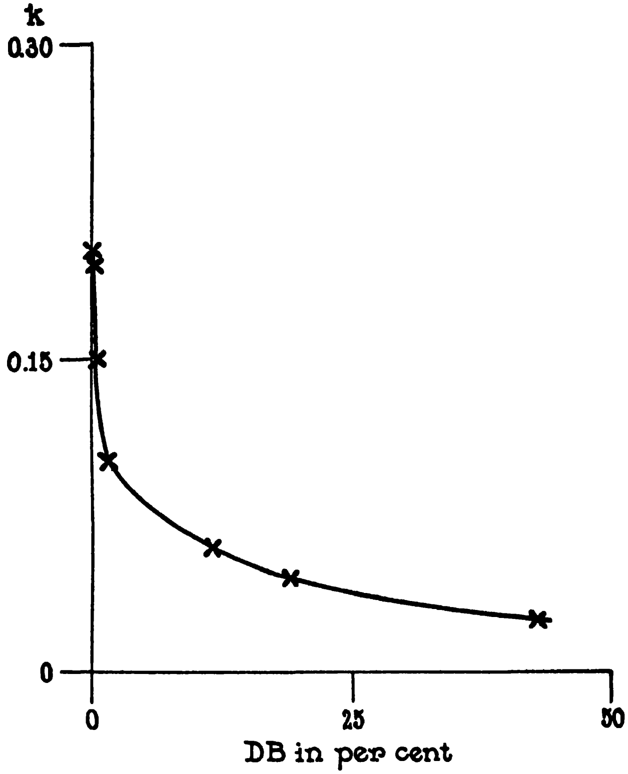

Fig. 3. Curve showing the relation of the velocity constants (k), plotted as ordinates, to the concentrations of free base (DB), in per cent as abscissæ.

TABLE II.

The Relation of the Dye in the Film Just Outside the Protoplasmic Surface to the Dye in the Vacuole, at Different External pH Values.

The values of b are obtained by the equation $b = (k_1 - \frac{1}{t}\log\frac{a}{a-x}) + k_1$ where k_1 denotes the velocity constant of the diffusion of the dye into or out of the living cell of *Nitella* where dye is present on one side of the protoplasmic surface only (the value of k_1 is assumed to be 0.37); where a denotes the initial concentration of dye in the vacuole; x the amount of dye that has diffused out of the vacuole at time t; and where b is a constant expressing the concentration of DB in the film as a fraction of the concentration of DB in the vacuole throughout the process at any one external pH value. Knowing the values of b, the values of y are obtained by the equation $y = b\ (a - x)$ where y denotes the DB in the film, $a - x$ the DB in the vacuole. Values of y thus obtained are relative values, since the given observed values of $a - x$ represent the "total dye" (DB + DS). Calculation is made with a 20 inch slide rule.

pH 5.4				pH 5.7				pH 6.0				pH 6.8			
$a - x$ obs.	k	b	y when b = 0.45	$a - x$ obs.	k	b	y when b = 0.47	$a - x$ obs.	k	b	y when b = 0.59	$a - x$ obs.	k	b	y when b = 0.73
M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5
4.83	0 215	0.42	2.17	4.83	0.215	0.42	2.27	5.59	0.152	0.59	3.30	6 21	0.107	0 71	4 55
3.05	0.208	0.44	1.37	3.28	0.192	0.48	1.54	4.00	0.148	0.60	2.36	4.83	0 107	0.71	3.53
2.15	0.189	0.49	0.97	2.31	0.179	0 52	1.09	2.76	0.153	0.59	1 63	4.14	0 094	0 75	3 02
								1.40	0 151	0.59	0 83	2.76	0.092	0 75	2.02
Average....		0.45				0.47				0.59				0.73	

pH 7.5				pH 7.8				pH 8.2			
$a - x$ obs.	k	b	y when b = 0.84	$a - x$ obs.	k	b	y when b = 0.88	$a - x$ obs.	k	b	y when b = 0.93
M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5
6.14	0.055	0.85	5.16	6.56	0.041	0.89	5.78	6.90	0.030	0.92	6.42
5.00	0.067	0 82	4.20	5.87	0.043	0.89	5.16	6.80	0.023	0 94	6.32
3.80	0.064	0.83	3.19	3.80	0.046	0.88	3 35	5.52	0.023	0.94	5.14
3.28	0.055	0.85	2.76	2.59	0.049	0.87	2.27	4.49	0.025	0 93	4.18
Average......		0.84				0.88				0.93	

0.3. Extrapolation has been attempted by various methods but with such a curve it is very difficult to obtain any reliable result. We may assume, however, that we are not too far from the true value if we take the maximum value of k to be 0.37.

If we solve for the values of b in the above equation, we find that they remain fairly constant for each external pH value, but they increase with an increase in the external pH value as shown in Table II. It may be stated here that the values of b are the same whether $a - x$ represents the "total dye" or DB.

Knowing the values of b and $a - x$, we may calculate the values of y by means of the equation: $y = b\ (a - x)$ for any value of $a - x$ as shown in Table II.

In calculating the values of y, the observed values of $a - x$ (Table II) representing the "total dye" in the sap are used for convenience. Since we are interested primarily in the relative values of y, such values will give us the desired information. It is needless to state that if the values of DB in the sap were used instead of those of the total dye (DB plus DS), the values of y would be considerably lower than those given in Table II, but the ratio of one value of y to another would remain unchanged.

At each external pH value the values of y are found to increase with increase in the value of $a - x$. If we take a fixed value of $a - x$ and compare the values of y at different pH values, we find that the value of y increases with an increase in the external pH value.

In order to bring out clearly the effect of y on the velocity constant of exit we may return to the equation on page 90

$$k = k_1 - k_1 b$$

and substitute the value $b = \dfrac{y}{a - x}$ (see page 88). We then have

$$k = k_1 - \frac{k_1 y}{a - x}$$

It may be added here that the values of k are the same whether $a - x$ represents the "total dye" or DB.

Let us now consider the relation of y to the per cent[14] of DB in the film (as determined by the pH of the external solution). We shall take for convenience the values of y where $a - x$ is 4.5×10^{-5} at different external pH values and take the percentage of DB as calculated from the distribution of DB between chloroform and water at different pH values of the external solution.

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[14] The discussion of the apparent dissociation constant is given in detail in the paper referred to in Foot-note 3.

Let us first see if the values of y at different pH values are proportional to the values in per cent of DB obtained from the dissociation[8] curve of the dye (which gives the DB in per cent calculated from the data obtained by the experiments on the distribution of the dye between chloroform and water). If we take for convenience the value 4.5×10^{-5} M for $a - x$ and find the value of y at pH 7.8 at which pH value 20 per cent of the dye is in the form of DB (according to the dissociation curve) we are able to calculate the values of DB in per cent on the basis of the values of y at other pH values (since we know the values of y for this fixed value of $a - x$), by the following equation.

$$\frac{y_1}{y_2} = \frac{m_1}{m_2}$$

when y_1 = the value of y at pH 7.8 = 4×10^{-5} M.
y_2 = the value of y at another pH value, say pH 7.5 = 3.8×10^{-5} M.
m_1 = 20 per cent.
m_2 = the DB in per cent at pH 7.5.

By substituting we get

$$\frac{4 \times 10^{-5}}{3.8 \times 10^{-5}} = \frac{20}{m_2}$$

$$m_2 = 19 \text{ per cent}$$

Where the values of DB are thus obtained for different pH values, they are found to be higher than the values of DB of the dissociation curve.

Since the values of y do not appear to be directly proportional to the values of DB in the dissociation curve, we may look for another relationship. If we plot the values of $\dfrac{\text{per cent DB in external film}}{y}$ (at varying pH values of the external solution) against the per cent of DB in the film (z), we get a line which is fairly straight, as shown in Fig. 4. This indicates a relation corresponding to Langmuir's[15] equation for adsorption,

$$y = \frac{m\,n\,z}{1 + zm}$$

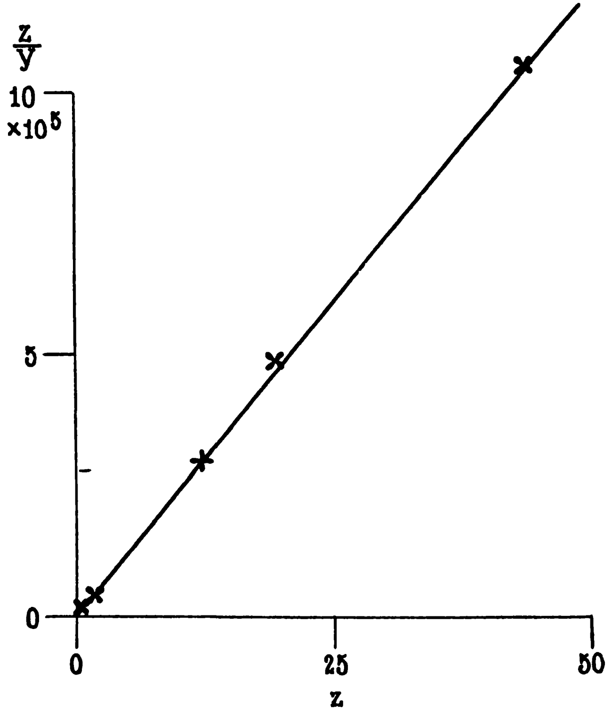

FIG. 4. Graph showing that Langmuir's equation $y = \frac{mnz}{1 + mz}$ (in which z = DB) may be applied to the process of the exit of the dye since the graph is a straight line. Ordinates represent $\frac{z}{y}$, and the abscissæ represent z.

where y is the substance adsorbed by a fixed concentration of an adsorbent, z is the concentration of the solution at equilibrium, and m and n are constants.

This might be regarded as indicating that the velocity constant (k) of the exit of the dye depends on the value of y which represents the amount of DB adsorbed by the protoplasmic surface from the film of external solution just outside the surface. But the applicability of this equation does not necessarily mean that we have to do with adsorption. For example, as Hitchcock[16] has pointed out, a similar relation applies if we have to do with a reversible chemical reaction where one of the reactants has a constant value.

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[15] Langmuir, I., *J. Am. Chem. Soc.*, 1918, xl, 1368.

It may be objected that if y represents the amount of DB adsorbed at the surface it will not be a constant fraction of $a - x$ during the entire process of exit of the dye but will be relatively greater during the latter part of the process. From the results of calculations which neglect this factor it is evident that it is not one of sufficient importance to effect any material change in the calculations here given.

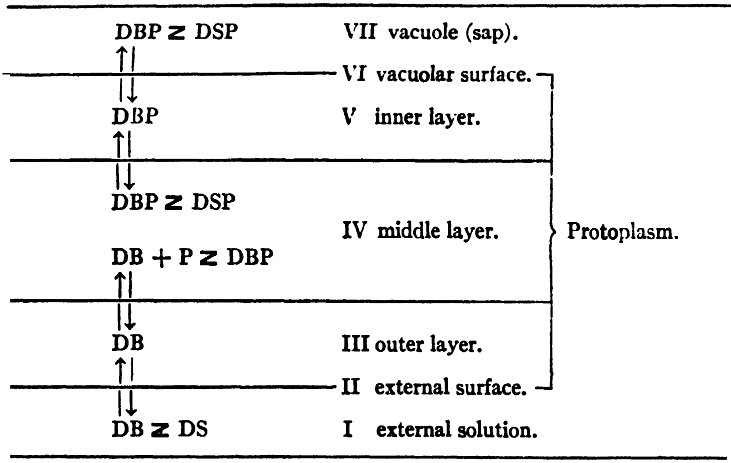

Diagram B. The cell wall is omitted for convenience.

If the surface forces constrain the molecules of DB so that they are not free to diffuse we meet with a difficulty. This difficulty would disappear if a constant fraction of the molecules is so constrained since that would merely lead us to divide the values of y by a constant factor.

The writer does not wish to lay any emphasis upon the fact that adsorption might possibly explain the relations observed but prefers to give the formula as a purely empirical one leaving the interpretation to future research.

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[16] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–26, viii, 61.

The preceding discussion of diffusion applies whether DB is in the form of undissociated molecules or ions.

Let us now consider the hypothesis that the rate of penetration and likewise of exit of dye from the vacuole is controlled by a chemical combination between DB and a constituent of the protoplasm. For this purpose we may modify Diagram A to conform to Diagram B or C.

Equilibrium between the two forms of dye is designated by the sign $\rightleftharpoons$ and diffusion by arrows $\rightleftarrows$. The entire mechanism represents a reversible process. Let us first take up Diagram B. DB can pass through III but DS cannot. As DB enters IV, it reacts with P, a protoplasmic constituent to form DBP. This form of dye compound, DBP, enters into equilibrium (according to the apparent dissociation constant) with another form of dye compound, DSP, which may represent a tautomere, or a complex compound. The ratio of $\frac{\mathrm{DB}}{\mathrm{DS}}$ and that of $\frac{\mathrm{DBP}}{\mathrm{DSP}}$ in IV depends on conditions in the protoplasm (pH value, solubility, etc.). DB, DS, and DSP are unable to pass through V, while DBP can pass through V but not through III. When DBP enters the vacuole it establishes an equilibrium with DSP, the ratio of $\frac{\mathrm{DBP}}{\mathrm{DSP}}$ being dependent on conditions in the sap, so that as long as the latter remain unchanged this ratio remains constant. The concentration of DBP in the sap is dependent on its concentration in the protoplasm, and on the concentrations of DB in all the parts described in the diagram. Thus, if the concentration of DB in III diminishes by its exit from III to I, then DB in IV decreases by its exit from IV to III, thus resulting in a decrease in DBP which in turn causes a corresponding amount of DBP to diffuse out from VII to IV.

We may consider a cell of *Nitella* in a solution as representing a heterogeneous system consisting of at least three phases: (1) the external solution, (2) the protoplasmic layer, and (3) the sap in the vacuole. If we venture to suppose that the protoplasm has non-aqueous layers at its outer and vacuolar surfaces, we shall consider the system to be composed of at least five phases.

The relation of the reaction $\mathrm{DB} + \mathrm{P} \rightleftharpoons \mathrm{DBP}$ in the protoplasm to the DB in the external solution may be made clearer if we consider the hydrolysis[17] of an ester in hydrochloric acid when the ester is distributed between HCl and benzene. As fast as the ester is hydrolyzed in hydrochloric acid, more ester passes in from the benzene. The rate of hydrolysis is controlled by the distribution coefficient, C, of ester between hydrochloric acid and benzene, since the lower the value of C the less ester diffuses from benzene to the hydrochloric acid in a given time. The equation for this process resembles that for a unimolecular reaction in a homogeneous system. The only difference is that this equation contains the correction for the partition coefficient C.

Thus in the case of the reaction DB + P ⇄ DBP, the rate may be assumed to be dependent on the amount of DB that passes into the protoplasm. If the concentration of DB in the external solution is raised, more DB will enter the protoplasm in a given time, and this will increase the rate of reaction. Exit of the dye from the protoplasm may also be explained on this basis. If there is no DB outside the cell, DB will come out of the protoplasm, and with the decrease in the concentration of DB in the protoplasm, the reaction DBP → DB + P will proceed faster; DB thus formed will continue to come out until there is no DBP in the protoplasm. But if the DB which comes out is not at once removed, a certain amount will diffuse back into the protoplasm, so that in a given time the decrease in the concentration of DB in the protoplasm will be less than when there is no DB outside. This will correspondingly retard the process DBP → DB + P, and hence diminish the rate of exit of DB.

So far as the relation of the reaction DB + P → DBP in the protoplasm to the DBP in the sap is concerned, the same explanation will hold. With an increase in the concentration of DBP in the protoplasm more DBP will diffuse into the sap. If DBP in the protoplasm decreases, on the other hand, DBP will tend to come out of the vacuole into the protoplasm. The rate of the reaction will depend on the concentration of DBP in the protoplasm. If for example the concentration of DBP in the sap is increased, causing a decrease in the amount of DBP diffusing out of the protoplasm into the vacuole, the concentration of DBP in the protoplasm will increase. This increase will retard the reaction DB + P → DBP. Thus the rate of reaction DB + P → DBP is controlled by the concentration of DB and DBP in the protoplasm. Increase in DB will hasten the reaction DB + P → DBP, while increase in DBP will retard it. The concentration of DB in the protoplasm depends on the amount of DB that enters or goes out of the protoplasm at a given time, and hence on the velocity of diffusion of DB through II or III in the diagram. The concentration of DBP in the protoplasm depends on the amount of DBP that goes out of the protoplasm into the vacuole, and the amount of DBP that enters the protoplasm from the vacuole, at a given time, and hence the rate of diffusion of DBP through V or VI in the diagram, B. It must be added here that the concentrations of DB and DBP are obviously interdependent.

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[17] Goldschmidt, H., and Messerschmidt, A., *Z. physik. Chem.*, 1899, xxxi, 235.

In view of the fact that the time curve for the hydrolysis of ester in hydrochloric acid, as described above, follows an equation similar to that of an irreversible unimolecular reaction in a homogeneous system, it is not surprising that we find in the case of penetration of dye into *Nitella* the unimolecular time curves for a homogeneous system.

Thus the analysis of the time curve of the exit of the dye may be made in this case by the use of the same equation as in the case of diffusion

$$\frac{dx}{dt} = k_1 (a - x) - (k_1 y)$$

where a denotes the concentration of DB at the start in the protoplasm, $a - x$ the amount left combined with protoplasm at time t, and y the amount of DB in the film of liquid just outside the external protoplasmic surface. The presence of DB in the film will cause some DB to diffuse back into the protoplasm, and thereby retard the decrease of DB in the protoplasm. This retards the rate of the reaction DBP → DB + P and hence it retards the exit of the dye from the vacuole.

What we actually measure is the concentration of the total dye (DBP and DSP) in the sap, and the value of $a - x$ is taken from the amount of the total dye in the sap at equilibrium. This method is justified since we are interested primarily in the relative values, and since we assume that the amount of the dye in the sap has a definite ratio to that of the dye in the protoplasm.

Another method of explanation is the following, as described in Diagram C.

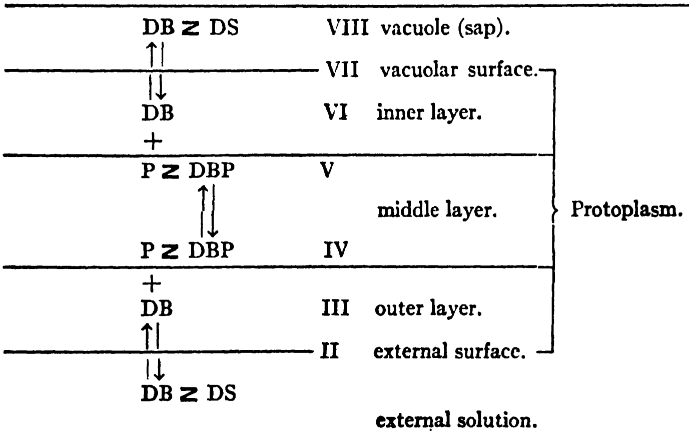

Diagram C. The cell wall is omitted for convenience.

DB can pass through III and VI. As DB enters at IV (the boundary between the outer layer and the middle layer) it combines with P of the protoplasm to form a complex compound DBP. DBP now diffuses from IV to V (the boundary between the middle layer and the inner layer) and DB is given off at V to VI. DB now diffuses into VIII. In I, II, VII, and VIII DB is in equilibrium with DS. The rate of DB + P → DBP is controlled by the concentration of DB and DBP in the protoplasm, and the concentrations of these substances are dependent on the amount diffusing in and out of the protoplasm in a given time. It is hardly necessary to undertake a detailed description of this diagram, as it closely resembles Diagram B. The only important differences are that the nature of the dye in the vacuole is not changed in this case, and that the reaction of DB + P → DBP in the protoplasm takes place at the boundaries IV and V. The latter may bring in complications to such an extent that we may have no justification for using an equation for a homogeneous reaction. Since so little is known in regard to this, the investigation of this question will be left to the future.

The experimental results thus far obtained do not show conclusively which one of the theories represents the mechanism. It may be possible that though there are reactions taking place between the dye and the protoplasmic constituents, the final result in both entrance of dye into and exit from the vacuole is dependent on the diffusion (see Section I) of the dye (see Foot-note 13).

A rough analogy to the passage of dye may be found in the case of entrance and exit of water into and from a reservoir, where the rate of inflow and outflow of the water depends on the conditions at the entrance and exit, and not on the conditions in the body of water between these two points.

V.

Rate of Exit When the pH Value of the Sap Is Changed.

The following experiments were carried out to determine if the theory thus proposed is supported by the observations on changes in the rate of the exit of the dye when the pH value of the sap is varied.

One lot of cells was placed in M/150 borate buffer solution at pH 8.5 containing 8.6×10^{-5} M cresyl blue and 0.005 M NH_4Cl; at the end of 5 minutes there was 8.6×10^{-5} M dye in the sap. The cells were then removed, wiped with a damp cloth, and placed in an M/150 phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes, the concentration of the dye in the sap was found to be 2.6×10^{-5} M.

A second lot of cells was placed in 0.005 M NH_4Cl at pH 8.5 M/150 borate buffers: at the end of 5 minutes the pH value of the sap had increased from pH 5.6 to 6.9. The pH value of the sap remained at 6.9 when such cells were placed in a buffer solution at pH 6.5 for 2 minutes.

A third lot of cells was placed in M/150 borate buffer solution at pH 8.5 containing 8.6×10^{-5} M cresyl blue: at the end of 45 seconds there was 8.6×10^{-5} M dye in the sap. The cells were now removed, wiped with a damp cloth, and placed in an M/150 phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes the concentration of the dye in the sap was determined and was found to be 5.9×10^{-5} M.

In all cases the experiments were carried out at 25 ± 0.5°C., and the solutions were constantly stirred and changed.

From these experiments it may be concluded that the rate of the exit of the dye from the cell sap is increased by presence of NH_3 in the sap which increases the pH value of the sap. Whether this increase in the rate is due to the increase in the pH value of the sap, or to the possible presence of NH_3 and consequent increase in pH value in the protoplasmic layer, or to NH_3 adhering to the cell surface, the writer is at present unable to determine.

The above observation is in agreement with the theory since the increase in the pH value would increase the concentration of DB in the sap and hence increase the concentration gradient, but in view of the fact that no appreciable changes in the pH value of the sap may be brought about without an injury to the cells, such a conclusion must necessarily be made with reserve. Furthermore, the fact that the rate of penetration is decreased,[18] while that of the exit of the dye is increased, when NH_3 enters the sap, does not prove that the dye enters the cell only in the form DB. As already suggested by the writer, in case DS enters,[19] the rate of penetration[20] may very well be decreased by the competition between the DS and the aqueous NH_3 for the substances (*viz.* salt of proteins or weak acid) in the protoplasm which may take place as NH_3 and DS enter the cell. The presence of aqueous NH_3 then would hasten the exit of DS from the cell, if the affinity of NH_3 for the cell substance is greater than that of DS.

SUMMARY.

Experiments on the exit of brilliant cresyl blue from the living cells of *Nitella*, in solutions of varying external pH values containing no dye, confirm the theory that the relation of the dye in the sap to that in the external solution depends on the fact that the dye exists in two forms, one of which (DB) can pass through the protoplasm while the other (DS) passes only slightly. DB increases (by transformation of DS to DB) with an increase in the pH value, and is soluble in substances like chloroform and benzene. DS increases with decrease in pH value and is insoluble (or nearly so) in chloroform and benzene.

The rate of exit of the dye increases as the external pH value decreases. This may be explained on the ground that DB as it comes out of the cell is partly changed to DS, the amount transformed increasing as the pH value decreases.

The rate of exit of the dye is increased when the pH value of the sap is increased by penetration of NH_3.

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[18] McCutcheon and Lucke (see Foot-note 9) believe that the decrease in the rate of penetration of a basic dye into *Nitella* with an increase in the pH value of the cell sap is a direct disproof of the theory that the dye combines with a protein in the cell.

[19] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235.

[20] When the pH value of the sap is decreased by an entrance of acetic acid the rate of penetration of dye is either increased or decreased, depending on the condition (probably of the protoplasm) of the cell.

FURTHER STUDIES ON THE INHIBITION OF CYPRIDINA LUMINESCENCE BY LIGHT, WITH SOME OBSERVATIONS ON METHYLENE BLUE.

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In a previous paper (1924-25) I have shown that a filtered luminescent mixture of *Cypridina* luciferin and luciferase has its luminescence suppressed (inhibited) by light from a carbon arc in 2 or 3 seconds with 15,000 foot candles illumination. The action of the light is upon the luciferin and not upon the luciferase. I have reported the suppression as partially reversible, the luminescence returning slightly in the dark, but am now inclined to regard this effect as apparent, due to better dark adaptation of the eyes. The inhibiting wave-lengths are in the blue-violet region, $\lambda = .46\mu$ to $\lambda = .38\mu$. Consequently one finds no suppression of luminescence by red, orange, yellow, or green light even after prolonged exposure.

The present paper deals with three further aspects of inhibition of luminescence by light, namely (1) photodynamic action of dyes; (2) influence of oxygen; (3) influence of H ion concentration on the inhibition.

1. *Photodynamic Action of Dyes.*—As the dye sensitization of many photochemical reactions is well known—notably that of the photographic plate by dicyanin, pinacyanol, erythrosin, etc., and the photodynamic action of acridine, eosin, etc., on living tissues, enzymes, and antibodies— it is not surprising to find a similar effect of dyes on the inhibitory action of light on *Cypridina* luminescence.

I have said that inhibition is brought about by blue (.46 to .38μ) light but not by red, orange, yellow, or green light. However, if we add to the luciferin–luciferase mixture one of a number of dyes, then we find that red, orange, yellow, or green light will inhibit the luminescence in a few seconds. The wave-length of light which will inhibit

Cypridina luminescence in the presence of sensitizing dye depends, of course, on the position of the absorption band of the dye, only those wave-lengths inhibiting which are absorbed. The converse is not necessarily true, that if a dye possesses an absorption band it is a photosensitizer with respect to that light. There may be or may not be sensitization.

A number of dyes have been tested by a student of mine, Mr. A. Hunsberger, Jr. The method is this. In a dark room, light from a carbon arc in a dark house passes through 6 cm. of water and is condensed to a small beam which strikes the middle portion of a narrow test-tube containing the luminescent mixture of *Cypridina* luciferin and luciferase. The light beam can be colored by light filters and cut off instantly by a photographic shutter. The luminescence of the exposed area of the test-tube is then compared with the non-exposed regions above and below.

Wratten gelatin filters were used to obtain light of a known range of wave-lengths. As the percentage transmission of the filters varies, some being much denser than others, it is impossible to select filters that will permit equal amounts of nearly monochromatic light to pass. In fact I have selected filters of high transmission which begin to absorb strongly at some definite wave-length (No. 15, 22, 29, 88) or those with broad transmission bands (No. 61) rather than the denser monochromatic filters, in order that the exposure need not be too long. 15 seconds was selected as a convenient time.

The filters are:

No. 61 green; over 10 per cent transmission .50 to $.57\mu$.
" 15 yellow; " 10 " " " .52 " $.70\mu$ and beyond.
" 22 orange; " 10 " " " .55 " $.70\mu$ " "
" 29 red; " 10 " " " .62 " $.70\mu$ " "
" 88 infra-red; no visible transmitted except 5 per cent at $.70\mu$.

The dyes tested are given in Table I.

Allowing for the unequal transmission of the filters and the widening of the absorption bands of the dye with increase in concentration, there is undoubtedly an agreement with the rule that the dyes sensitize only for that wave-length of light which they absorb.

It can also be shown that the inhibiting action of green light in presence of eosin is upon the luciferin and not the luciferase, just as in the case of violet wave-lengths acting without sensitizer.

I think we may predict with fair certainty that Ctenophores, the inhibition of whose luminescence by light is so well known, will also be sensitized by photodynamic dyes.

2. *Influence of Oxygen.*—It must be recalled that luminescence only occurs if luciferin, luciferase, and oxygen are together in solution, but that luciferin will oxidize without luminescence if luciferase is absent.

It seems most probable, therefore, that light acts by causing rapid oxidation of luciferin without luminescence. Consequently the area exposed to light, of a luminescent mixture of luciferin and luciferase in a test-tube, will not luminesce so brightly because some of the luciferin has been photochemically oxidized. This view can be tested by completely exposing luciferin solutions to light in absence of oxygen (by evacuation or bubbling of pure hydrogen), with a control tube illuminated in presence of oxygen, and then mixing both tubes with luciferase. The luciferin exposed in absence of oxygen gives a bright light, while the control in presence of oxygen gives no luminescence or a very faint one. There is no inhibition in absence of oxygen.

TABLE I.

Dye.	Structure.	Absorption in weak and stronger sol.	Photosensitization in 15 sec.
		μμ	
Fluoresceine.		475–502 450–505	Negative.
Eosin.	K tetrabromfluoresceine.	500–530 480–540	Green, yellow.
Erythrosin.	K or Na tetraiodofluoresceine.	500–530 480–560	" "
Rose bengale.	K or Na 4 iodo 2 chlorfluoresceine.	530–550 480–560	" "
Cyanosin.	K methyl ether of 4 Br 2 Cl fluoresceine.	500–545	" "
Acridine or aniline red E 103.		480–530 430–570	" "
Methylene blue.	Tetramethyl thionin HCl.	650–680 560–680	Yellow, orange, red.

I find also that the photosensitive dyes will not sensitize the inhibition of luminescence from luciferin in green or yellow light in absence of oxygen. In this respect the phenomenon agrees with photodynamic dye action in tissues. There is no poisonous action of eosin on enzymes or organisms in light in absence of oxygen (von Tappeiner, 1909).

The *dye* simply acts by making wave-lengths effective which would not be effective in its absence. The *light* acts by rapidly oxidizing luciferin.

The manner in which the photosensitizers make wave-lengths photochemically effective is not well understood. Perhaps the first question to be asked is whether the sensitizer undergoes any change. We may suppose the eosin to oxidize the luciferin in presence of light, itself undergoing reduction to a leuco body. Accordingly I have exposed mixtures of luciferin and eosin to white light (8600 foot candles) and also to green light, for from 4 to 7 minutes, but have never observed any indication of the decolorization of eosin, although it is known that eosin is affected by light (Gros, 1901). There is also no indication of the decolorization of methylene blue and luciferin exposed to white light (8600 foot candles) for 4 minutes, although the oxidative action of the light on luciferin is greatly increased by the presence of these dyes. If any change occurs in the dye it involves no color change or is momentary. Under proper conditions, however, methylene blue is affected by light, as described below.

3. *Influence of H Ion Concentration.*—I have often compared luciferin to leuco methylene blue and its oxidation to the oxidation of leuco methylene blue with formation of the blue dye. Expressed as a reaction the change would be

$$MH_2 \text{ (leuco methylene blue or methylene white)} \rightleftarrows M\text{(methylene blue)} + H_2$$

$$LH_2 \text{ (luciferin} \rightleftarrows L \text{ (oxyluciferin)} + H_2.$$

$$H_2 + O = H_2O$$

In view of the effect of light on luciferin oxidation it is interesting to note that methylene white oxidation is affected by light also. This was observed by Clark (1925), and the effect can be very nicely seen by reducing methylene blue with Zn dust and dilute acid, pouring the colorless solution into a narrow test-tube and exposing the middle portion of the colorless solution to the condensed beam from a carbon arc lamp, first passing the beam through a water layer to remove its heat. Blueing will occur in the illuminated region in a few seconds. Oxygen is of course present in this experiment, but I can confirm Clark's observation that blueing will occur *in absence of oxygen* after reduction by platinized asbestos and hydrogen. *However, the solution must be acid.* After removal of oxygen and reduction by $Na_2S_2O_4$, blueing of methylene white in light will also occur, provided the solution is acid enough and that not too much $Na_2S_2O_4$ has been added. Neutral and alkaline solutions will not turn blue in light under the same conditions. There seems to be a shift in equilibrium of the methylene blue $\rightleftarrows$ methylene white system toward the side of oxidation in the light.

If methylene blue is reduced by Pt asbestos and hydrogen in two tubes, one of M/50 HCl, the other of M/50 NaOH, and the tubes shaken slightly to dissolve a little oxygen it can be easily observed that the methylene white oxidizes much more quickly in the alkaline tube, which becomes blue as compared to the acid tube, only faintly bluish—a well known phenomenon. On now exposing the two tubes to a beam of light, there results a blue band in the acid medium and a colorless band in the alkaline medium. We have acceleration of oxidation in acid and of reduction in alkali in light. I have observed the same thing when NH_4SH or H_2S is used as reducing agent and also when $Na_2S_2O_4$ is employed.

If just enough $Na_2S_2O_4$ is added to decolorize methylene blue in M/50 HCl and the colorless tube is exposed to light, blueing will occur; but if a little more $Na_2S_2O_4$ is added no blue band occurs in light. However, upon shaking with air until some of the $Na_2S_2O_4$ is removed by oxidation, a blue band now appears in light. Reducing with $Na_2S_2O_4$ in an alkaline medium (Clark and Lubs buffer, M/20 H_3BO_3, KCl, NaOH, pH = 10) and exposing to light, we observe no change; but if shaken with air till partly blue and then exposing to light, a colorless band appears.

This colorless substance is methylene white and not a colorless oxidation product of methylene blue, because by thorough shaking with air the blue color will return again completely.

We may sum up the behavior of methylene blue in light as follows: Methylene white in presence of reducing agents will turn blue in absence of oxygen if the solution is acid enough but not in neutral or alkaline solutions. In presence of some oxygen and reducing agent[1] acidity favors the change to blue (oxidation) while alkalinity favors the change to colorless (reduction). Without reducing agent, methylene blue will be rendered colorless by light slowly in fairly alkaline solution (M/50 NaOH) but not in M/10 Na_2HPO_4 (pH = 9) or in distilled water.

Indigo carmine does not behave like methylene blue under the influence of illumination.

If the luciferin–oxyluciferin system is to behave like methylene blue, we should expect inhibition of luminescence in light in acid medium containing some oxygen and reducing agent[1] (like $Na_2S_2O_4$). Under these conditions oxidation to oxyluciferin would be favored and less luciferin remain to luminesce with luciferase. Hence a dark band should appear in a luminescent tube after illumination. On the other hand, in alkaline medium a luminescent band should appear after illumination, since reduction would be favored and more luciferin accumulate in the previously illuminated region.

However, I have been unable to observe a more luminescent band after illumination in solutions of any reaction. If luciferin is prepared in a series of buffer solutions and a little luciferase added we get the following results in light-exposed and dark regions of the tube. No reducing agent is present.

Buffer solution and pH.	Luminescence in dark.	Luminescence in light.
M/20 K H phthalate = 4.	Very faint.	Very slowly inhibited.
M/20 K H phthalate, NaOH = 5.6	Faint.	" " "
Sea water = 8.	Good.	Rapid inhibition.
M/20 H_3BO_3, KCl, NaOH = 9.	Faint, fading quickly.	" "
M/20 " " " = 10.	" " "	" "

It will be noted that inhibition, indicating oxidation of luciferin, always occurs no matter what the reaction, acid or alkaline.

If $Na_2S_2O_4$ is now added to the above tubes to remove the oxygen the luminescence disappears and exposure to light in absence of oxygen never causes luminescence to return. If the tubes are shaken slightly to dissolve oxygen, luminescence will return and then exposure to light gives the same results as recorded in the table in the absence of any reducing agent. Light exposure always results in inhibition which is more rapid the more alkaline the medium and is also more rapid in presence of the $Na_2S_2O_4$ (or its oxidation products) than previously. It is as if the oxidation products of $Na_2S_2O_4$ accelerated the effects of light, as they do in the case of methylene blue. I have never observed a more luminescent band in the region previously exposed to light.

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[1] Or its oxidation product.

We see that the behavior of luciferin in light is only in part similar to that of methylene blue. One always obtains acceleration of oxidation of luciferin by light and not acceleration of reduction under the same conditions (alkaline medium) necessary for the phenomenon in methylene blue. Perhaps it is pushing the analogy too far to expect that the methylene white—methylene blue and the luciferin–oxyluciferin systems will behave in exactly the same way after exposure to light.

SUMMARY.

1. Eosin, erythrosin, rose bengale, cyanosin, acridine, and methylene blue act photodynamically on the luminescence of a *Cypridina* luciferin–luciferase solution. In presence of these dyes inhibition of luminescence, which without the dye occurs only in blue-violet light, takes place in green, yellow, orange, or red light, depending on the position of the absorption bands of the dye.

2. Inhibition of *Cypridina* luminescence without photosensitive dye in blue-violet light, or with photosensitive dye in longer wave-lengths, does not occur in absence of oxygen. Light acts by accelerating the oxidation of luciferin without luminescence. Eosin or methylene blue act by making longer wave-lengths effective, but there is no evidence that these dyes become reduced in the process.

3. The luciferin–oxyluciferin system is similar to the methylene white–methylene blue system in many ways but not exactly similar in respect to photochemical change. Oxidation of the dye is favored in acid solution, reduction in alkaline solution. However, oxidation

of luciferin is favored in all pH ranges from 4 to 10 but is much more rapid in alkaline solution, either in light or darkness. There is no evidence that reduction of oxyluciferin is favored in alkaline solution. Clark's observation that oxidation (blueing) of methylene white occurs in complete absence of oxygen has been confirmed for acid solutions. I observed no blueing in light in alkaline solution.

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TEMPERATURE CHARACTERISTICS FOR DURATION OF AN INSTAR IN CLADOCERANS.

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I.

The analysis of growth phenomena through determinations of their relations to temperature requires measurements of the rates of development within stages morphologically well defined and exhibited by individuals genetically uniform and comparable. The duration of an adult instar in Cladocera parthenogenetically produced within a single clone is in several respects a very suitable object for such measurements. It is not altogether clear, however, whether the incidence of ecdysis is directly determined by the growth of the female, or by the full development of her young which ecdysis liberates. It is proposed to investigate this point more fully in subsequent experiments, but the latter interpretation is probably the correct one, for mothers about to release young, if transferred to a considerably higher temperature, will release the young properly but they themselves become caught in the moult which is being cast. In other words the young, at the higher temperature, have reached full development and have apparently caused the mother to moult before the carapace was in proper condition to be cast. For the present it is sufficient to note that the period of growth or development utilized for measurement bears relations to temperature of a kind very closely comparable with those shown by relatively simple vital processes (Crozier, 1924–25, *a*, *b*), and obtained likewise for the velocity of development within a clearly delimited stage in *Drosophila* (Bliss, 1925–26).

Comparison of several species of cladocerans reared in the laboratory for many generations under similar conditions should reveal specific differences, should such be present, and might be taken to suggest a means for the physiological comparison of related forms.

Three species of Cladocera were employed. Two of these, *Moina macrocopa* and *Simocephalus serrulatus*, belong to the family Daphnidæ; the third species, *Pseudosida bidentata*, belongs to the family Sididæ. A few individuals of *M. macrocopa* and of *P. bidentata* were sent me by Dr. A. M. Banta, both forms having been reared parthenogenetically for many generations in his laboratory. *M. macrocopa* was originally collected near Cold Spring Harbor, Long Island, and it occurs there in small exposed ponds from April through October. The population in the ponds increases rapidly to a maximum in June and the form is found in slightly diminished numbers until the middle of October. The clone of *P. bidentata* that was used was started from individuals collected in Florida in February; I have not taken this form in the north. *S. serrulatus* was collected near Cambridge, Massachusetts, early in October. In general it is a species that is quite wide spread and it may also be taken during any month of the year, usually reaching large numbers towards the last half of April and the first of May, continuing with few individuals during July and August, and reaching a maximum in October.

II.

In the laboratory, reproduction in all species was exclusively parthenogenetic, starting from one female and thus insuring material genotypically identical throughout. The stock cultures were reared at room temperature in an especially devised culture medium (Banta, 1921). The animals used in any one experiment were usually the first or second generation descendants of one individual. Such animals were reared with ample food, so that they would produce average sized to large broods, brood size being a good indication of the vigor of the stock. For precise work it is necessary to study a stage of development having sharply marked beginning and end. In this case one adult instar, usually the second, was chosen as the period to be measured. The successive instars of a given animal, when reared at the same temperature, do not show any appreciable differences in length. The beginning of an adult instar is marked by the release of active young daphnids from the brood chamber of the mother, and the end of the instar is marked by the release of the succeeding brood of young. In *M. macrocopa* the release of the young, the moulting of the mother, and the passing of a new clutch of eggs to the brood chamber occupies from 2 to 6 minutes at room temperature. The time for this series of events is slightly more variable in *P. bidentata*, but not so variable as in *S. serrulatus* in which the time from release of young to egg laying varies from a few minutes to half an hour in extreme cases.

As the adult females to be tested were nearing the end of an instar they were transferred to individual bottles and placed in constant temperature cabinets. A bottle of food was placed beside a bottle containing a female. The termination of an adult instar, that is, the appearance of young in the bottle, is foreshadowed by the darkening and coalescing of the eye rudiments of the embryos. Observation of the mothers was made at frequent intervals (15 minutes to an hour, depending on the temperature), and the time of the release of the young noted. The mother was transferred to the adjacent bottle and allowed to remain there until the next brood of young was released. When it was impossible to watch for the termination of the instar in question at intervals of an hour or less, those mothers that probably would soon liberate young were examined microscopically and an estimation made of the probable time of release of her young (based upon the condition of the eye pigment of the embryos). This estimated time was further checked by observing the developing eggs of the next brood and noting the stage of segmentation of the egg. If the elapsed time was too long the animal was of course discarded.

Four temperature cabinets were used, in each of which the temperature to be maintained could be raised or lowered at will. The cabinets were heated by carbon filament bulbs controlled by a mercury thermoregulator. The different regions (shelves) of the cabinet varied in temperature depending on the distance from the heating unit, but the temperature of each bottle was taken directly. The extreme variation within a given bottle during an experiment was ± 0.5°C. Slightly different rates of general metabolism are found for the females producing male broods and those producing female broods (Banta and Brown, 1924–25). This has been determined for *M. macrocopa;* the male broods are released on the average later (half an hour, more or

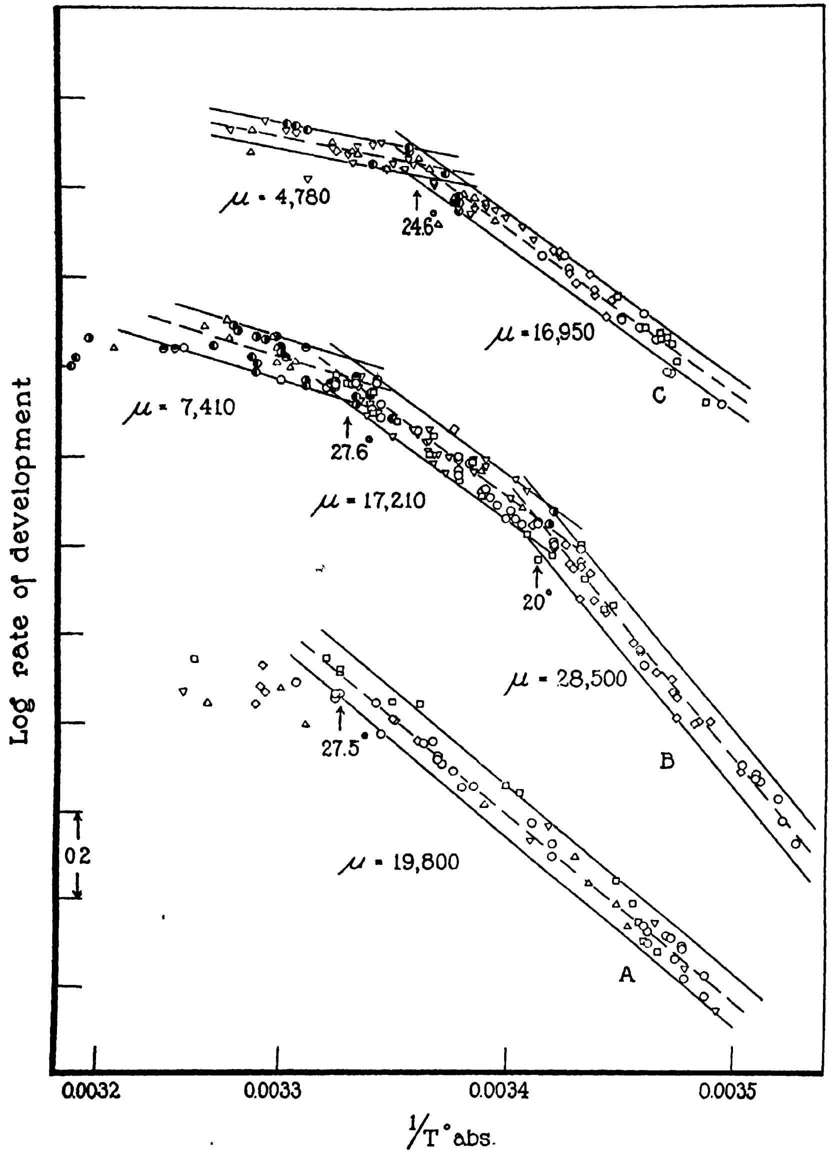

Fig. 1. Graphs for rates of development plotted against reciprocals of the absolute temperatures. *A*, *Pseudosida bidentata*. *B*, *Moina macrocopa*. *C*, *Simocephalus serrulatus*. Each point represents an individual animal and the different symbols in each graph denote a single experiment. The values of μ are given opposite the segments of the graphs, and the critical points are indicated by arrows, with the centigrade temperatures given for these points. (Rate of development = 10,000 ÷ time in minutes; one unit on log scale = 0.2.)

less) than the female broods. It has not been possible to employ numbers of animals sufficient to remove this source of variation, which would at most amount to between 1 and 2 per cent of the total observed time.

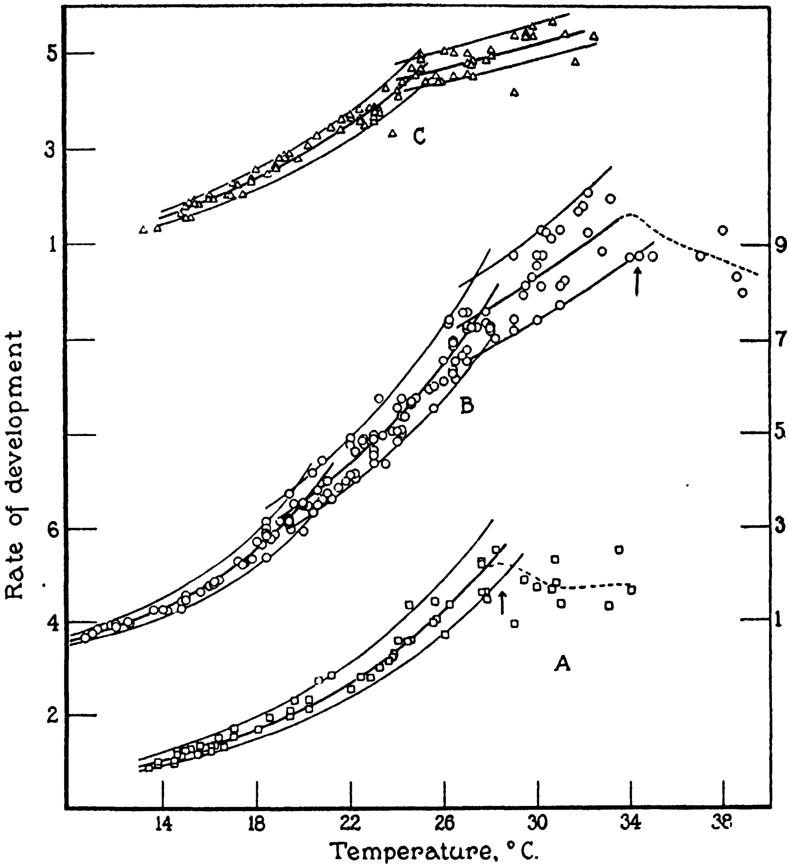

FIG. 2. *A*, *P. bidentata.* *B*, *M. macrocopa.* *C*, *S. serrulatus.* The rates of development are plotted against centrigrade temperatures, and the points are entered without distinguishing individual experiments. The lighter lines and the heavier lines are transposed from those in Fig. 1.

III.

Fig. 1 gives the graphs for the rates of development in the three species. The logarithm of the rate is plotted against the reciprocal of the absolute temperature. Each point on the graph represents an individual animal and the different symbols in each graph denote a single experiment. The experiments were conducted at different times between January and May of the same year to examine the possibility of seasonal rhythm. It is clear that there is no secular drif due to such a cause.

The graph for *S. serrulatus* (Fig. 1, *C*) shows a break at 24.6°C. The slope of the line below this temperature gives a temperature characteristic $\mu = 16{,}950$. In the neighborhood of 15° and below there are insufficient data to determine the exact slope and the presence or absence of a break at this point. At 24°C. there is a point (upright triangle) which has a lower rate. And again at 29°C. there is a point (inverted triangle) which has a lower rate. In neither case was there sufficient latitude in the observations to bring these points within the range of variation of the other points on the graph. These may be individuals abnormal either through starvation or age. The character of the line above 32° has not yet been determined. The slope between 24.6° and 32° yields $\mu = 4{,}780$.

The graph for *M. macrocopa* (Fig. 1, *B*) is based on more animals than are the other two graphs. There are three evident breaks or critical temperatures. The first break is at 20°, the second at 27.6° and the third somewhere near 33°. The steepest slope, with $\mu = 28{,}500$, is found between 11° and 20°. The slope of the line between 20° and 27.6° gives $\mu = 17{,}210$, which agrees fairly well with that for *S. serrulatus* below 24.6°. Above 27.6° there is a distinct lessening of the slope, the value for μ dropping to 7,410. Above 33° the points become irregular and the curve flattens out into an almost horizontal position. It is interesting to note that even at 38° + the females of this species produce apparently normal young. The range of variation, as indicated by the limiting lines, is quite uniform for the different parts of the graph, being slightly greater above 27°; this may be due to the faster rate of development and the correspondingly decreased accuracy of the observations.

The graph for *P. bidentata* (Fig. 1, *A*) is strikingly different from the other two. This graph consists of one straight band, having the value of $\mu = 19{,}800$, extending from 14° to 27.5°. Beyond 28° the points scatter and the curve assumes a position approximately parallel to the temperature axis. There is thus but one break, at 27.5°, in the graph for *P. bidentata*.

Inspection of graphs such as that for *M. macrocopa* in Fig. 1 may lead to the superficial suggestion that it is preferable or possible to draw through the plotted points a curve, rather than several straight lines. The fact that it is impossible to fit a single simple curve to the graphs for *S. serrulatus* and *M. macrocopa* is perhaps shown more clearly in Fig. 2. In this figure the rate of development, that is the reciprocal of the time taken to complete an instar, is plotted against centigrade temperature. The lighter limiting lines and the heavier lines in this figure are transposed from those in Fig. 1. It is impossible to fit a single smooth curve through the points for either of these species. The points fall respectively into two and three cusps (when the irregular individuals above 33° for *M. macrocopa* are excluded). The points for *P. bidentata* form a smooth curve from 14° to 27.5°, this whole range yielding a constant value of μ.

The range of variation of course increases with an increase in temperature. It will also be noticed that the latitude of variation for *S. serrulatus* is much smaller than for *M. macrocopa* and for *P. bidentata*, in spite of the fact that the end-points for an instar in *S. serrulatus* are more variable, intrinsically, than in the other two species. This would seem to indicate that the latitude of variation is specifically determined, but is not a property of the system controlled by the nature of the process which determines the temperature characteristic.

IV.

A comparison of the values for μ with similar values obtained for rates of development in other arthropods shows some remarkable similarities, and may be taken to indicate a similar chemical control in the several instances. Bliss (1925–26) obtained a value for μ of 16,850 for the prepupal development of *Drosophila melanogaster*, from 16° to 25°. Crozier (1924–25, *b*), using data from Krogh, obtained a value of 16,850, 16° to 32°, for μ in O_2 utilization of *Tenebrio* pupæ, and Orr (1924–25) obtained a value for μ of 16,800, 1° to 15°, for the O_2 consumption in the prepupa and pupa of *Drosophila*. These values are of the same order as the 16,950 obtained for *S. serrulatus* between 15° and 25°, and the value 17,210 obtained for *M. macrocopa* between 20° and 28°. The value of μ for *M. macrocopa* below 20° does not correspond to the value obtained by Bliss with *Drosophila* below 16°, but it may be of the same nature (28,500) as the value 27,000 obtained from Krogh's data by Crozier (1924–25, *b*), below 22° in *Tenebrio* pupæ, that for *Drosophila* egg and larva between 10° and 20° and for *Drosophila* pupa at 15° to 20°, both of which gave $\mu = 27{,}000$ (Crozier, 1924–25, *b;* data from Loeb and Northrop). For *M. macrocopa* the temperature range 28° to 33° yields $\mu = 7{,}410$, which compares favorably with the value obtained by Bliss for prepupal development in *Drosophila* between 25° and 30° (*i.e.*, 7,100). Also it might be noted that the distribution of the temperature characteristics, with the exception of 4,780 for the upper portion of the graph for *S. serrulatus*, corresponds to peaks in the frequency graph for temperature characteristics given by Crozier (1925–26, *d*).

The breaks in Fig. 1, when rounded off to the nearest whole degree, are located at 15° (?), 20°, 25°, 28°, 30°, and 33°. Crozier has pointed out (Crozier, 1925–26, *c*) that such critical points are usually found in the neighborhood of 4.5°, 9°, 15°, 20°, 25°, 27°, and 30°. Setchell (1925) has pointed out that aquatic and land plants give definite points of critical temperature for anthesis, these points being at approximately regular intervals of 5 degrees from 5° to 30°. The critical points obtaining for the three species of Cladocera thus agree in a rather remarkable way with those found for vital phenomena in general.

A comparison of the three species of Cladocera used in these experiments shows some points of possible significance. The temperatures for *S. serrulatus* above which rate of development is relatively slower, *i.e.* lower value for μ, are 15° (?), 25°, and 30°, while the corresponding temperatures for *M. macrocopa* are 20°, 28°, and 33°. Thus *M. macrocopa* is, upon the basis of rate of development at least, enabled to take advantage of increasing temperature by producing broods in quicker succession than *S. serrulatus*. And conversely, on a lowering temperature, *M. macrocopa* is relatively more slowed up. As previously stated, *M. macrocopa* is typically a summer form while *S. serrulatus* occurs throughout the year but in greater abundance in the spring and autumn. *P. bidentata* cannot be compared so directly. This species maintains a constant $\mu = 19{,}800$ from 13° to 28°. This rate of increase with temperature is decidedly greater than that of the other two species above 20°. The distribution of *P. bidentata* is known to be southern and was studied during the winter, but its annual rhythm of abundance is not known at present.

SUMMARY.

1. The temperature characteristics for the rate of development during a well defined instar were determined for three species of Cladocera, and found to be of the same general magnitudes as those obtained for rates of development and of O_2 consumption in other arthropods.
2. Critical temperatures were found to occur at points most frequently critical in quite diverse vital phenomena as determined by abrupt changes in the relationship between rate and temperature.
3. A suggestion is made that, since the values of μ and the positions of critical temperatures obtained for the different species are not the same, some relation may exist between the occurrence of these forms in nature and their relative rates of development as controlled by temperatures.

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THE INFLUENCE OF LIGHT, TEMPERATURE, AND OTHER CONDITIONS ON THE ABILITY OF NITELLA CELLS TO CONCENTRATE HALOGENS IN THE CELL SAP.

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In connection with a general investigation of the absorption and utilization of mineral elements by plants, various observations on the cells of *Nitella clavata* were previously reported.[1,2] The primary object of the experimentation is to gain some additional insight into the fundamental processes of absorption in their relation to the nutrition of higher plants. Special attention has, therefore, been given to dilute solutions, comparable to soil solutions, and to the intake of mineral elements by various types of plant cells from the point of view of growth and metabolism, rather than that of permeability *per se.* Certain phases of the general problem which were previously outlined have now received further study and the development of a number of new methods of procedure has made it possible to obtain much more extensive and satisfactory data than heretofore. Reference to the recent work of Osterhout and his colleagues on *Valonia* and *Nitella* will be deferred until later in the discussion.

In our earlier experiments on *Nitella* certain preliminary data were obtained on the absorption of bromine. This element seemed to offer promise of being useful in studying the phenomena of absorption, since it is non-toxic or practically so in low concentrations, and is not normally present in the cell sap of these plants. At first the estimation of bromine was made by a colorimetric method, but this was not found to be satisfactory for quantitative work and it did not appear

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[1] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

[2] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, vi, 47.

that any technique so far described for determining bromine met our particular requirements. One of the writers (P. L. Hibbard), therefore, devoted considerable effort to devising a method suited to the purpose. Eventually, it was found possible to determine bromine in the presence of chlorine or iodine with the use of very small quantities of cell sap (1 cc.) and within a reasonably short time. The analytical procedure and the magnitude of the error, when this method is applied to plant sap, are discussed elsewhere.[3] Considering the very minute absolute quantities dealt with, the accuracy of the results was better than might be anticipated. As a rule errors other than those connected with the analysis limited the interpretation of the data, except in those cases in which very small concentrations of bromine were present in the sap, when the percentage of error in the analytical data was unavoidably high. Nearly all analyses were made in duplicate and the experiments were also duplicated or repeated. It is believed that due care has been taken to limit the conclusions in accordance with the significance and consistency of the results, as will be further indicated in the discussion of the specific data. Fortunately, the effects we sought to demonstrate were of large relative magnitude so that they stand out quite clearly. At the present stage of development, it is extremely doubtful whether anything would be gained by any considerable refinement of the experiments, which might involve an almost prohibitive amount of labor.

Cell sap only slightly contaminated was obtained by the method of breaking individual plant cells in the manner previously described. Cells used for this purpose were very turgid and varied in length from ½ to 3 inches. In addition to this method of obtaining sap, in a few experiments, sap was also prepared by expressing (by hand pressure) the masses of cells remaining after nearly all the large cells had been selected out. The sap prepared in this way and filtered is designated as "expressed sap" and that derived from individual cells as "cell sap." The different substances were present in the expressed sap in concentrations only about one-half those of the cell sap. Of course, there must have occurred a very appreciable dilution of the cell sap in the former case by water still adhering to the outer surfaces, even after shaking the mass of cells vigorously, but it is also possible that the many very small cells (not more than ¼ inch in length) contained sap of lower concentration than that of the sap in the large cells. Conclusions with regard to the general relations existing between the cell sap and the external medium would have been very similar if the expressed sap alone had been considered, but the results on the cell sap are obviously more definite and convincing, and, furthermore, the expressed sap cannot give an adequate idea of the extent to which certain elements may become concentrated in the vacuole.

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[3] Hibbard, P. L., *Ind. and Eng. Chem.*, 1926, xviii, 57.

In many of the experiments it was found convenient to use a general culture solution to which bromide was added as desired. This solution was buffered with phosphate and had the following approximate composition.

KH_2PO_4	5	milli-equivalents
$Ca(H_2PO_4)_2$	2	"
NaOH	6	"
pH	5.0–5.4.[4]	

During the course of experiments of extended duration the initial pH value of the culture solution usually increased by several tenths, whether as a result of selective absorption or because of the lime deposits frequently adhering to the outside surfaces of the cells. Solutions of the composition stated above were not found to show any apparent toxicity within the time of the experiments. Under favorable environmental conditions, masses of cells could be kept in such solutions over periods up to 2 months in as good a condition as in tap water. Bromides in a concentration of .005 molar were not toxic as far as could be observed and much higher concentrations produced only very slight injury, if any. Of course, in any solution, there occurs a gradual dying off of a certain number of cells, but this was not more noticeable with the experimental solution than with tap water for the periods in question. In both types of media, under appropriate conditions of light and temperature, much new growth took place in the course of a month or more. After 2 or 3 months, cells kept in these solutions showed evidence of injury, but a complete culture solution (plus $CaCO_3$) containing bromide was as favorable a medium as tap water, judging by the new growth obtained during the course of a year.

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[4] Loss of chlorine accompanied by injury was not found to occur unless the pH value was below 4.8. The marked loss of chlorine and injury beginning at about pH 4.4 is correlated by Pearsall (Pearsall, W. H., and Ewing, J., *New Phytologist*, 1924, xxiii, 1923) with the isoelectric point of the *Nitella* proteins. With regard to the pH value of the cell sap, the various treatments were not found to alter appreciably the normal value of 5.2.

TABLE I.

Comparison of Concentration of Br and Cl in Sap from Cells Exposed to Different Types of Media Containing KBr or KCl.

Composition of medium.	Concentration of Br or Cl in cell sap.	Period of exposure.
	m -EQ	
Buffer solution + KBr 5 0 m.-Eq.	22 7 Br.	6 days continuous illumination.
KBr 5.0 m.-Eq., no buffer	24 8 "	" " "
Buffer solution + KBr 5.0 m.-Eq.	57 0 "	17 days continuous illumination.
KBr 5.0 m.-Eq., no buffer	49 5 "	" " "
Buffer solution + KCl 5.0 m.-Eq.	127 5 Cl.	6 days continuous illumination.
KCl 5.0 m.-Eq., no buffer.	118 0 "	" " "
Buffer solution + KCl 5.0 m.-Eq.	136 2 "	17 days continuous illumination.
KCl 5.0 m.-Eq., no buffer.	140 5 "	" " "
Buffer solution + KBr 5.0 m -Eq.	20 5 Br.	6 days daylight.
Tap water + KBr 5.0 m -Eq.	11 3 "	" " "

pH of phosphate buffer solutions + KBr and of KBr solutions 5.0–5.4. pH of tap water + KBr 7.0 or above.

Other data prove the lessened absorption from tap water was, to a large extent, caused by the influence of the chlorine ions.

Later studies showed that the accumulation[5] of Br was very similar whether KBr was used alone or added to the buffer solution (Table I). It may be noted that the former solutions were not completely free of traces of calcium, because of contamination from surface deposits on the cells. Therefore, no conclusion can be drawn with regard to the effect of a complete absence of calcium from the solution, but it is evident that in dilute solutions of this character, relatively high in K, the calcium added to the solution had no striking effect on the accumulation of Br, and that the use of buffer salts was not influential in determining the course of absorption.

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[5] For convenience of discussion, we are using the word "accumulates" in the sense proposed by Osterhout, *i.e.* when a substance reaches a higher concentration in the sap than in the surrounding solution.

Most of the experiments were carried out in beakers or wide mouth bottles with a capacity of 3 or 4 liters. From 100 to 125 gm. of cells, drained free of excess water, were placed in 3 liters of solution. The mass of cells was previously washed thoroughly in distilled water. At the end of an experiment the cells were removed from the solution, first washed with tap water and then with distilled water, after which the sap was recovered as already indicated Except in experiments in which complete analyses were to be made, when several thousand cells were used, each sample of cell sap ordinarily represented several hundred cells and varied in volume between 2 and 4 cc. Because of the large number of cells which each sample of sap represented, errors resulting from the variability of individual cells were reduced sufficiently to permit satisfactory comparisons of different treatments Incidentally, it may be remarked that while the whole procedure is exceedingly tedious and time-consuming, no easier way of obtaining direct evidence concerning the composition of the cell sap has been suggested, and it is just this type of evidence which is most needed at the present time.

Temperature Effects.

In this series of experiments, we desired to obtain some indication of the temperature coefficient for the absorption of Br under controlled light conditions. Two double walled baths were constructed with arrangements for flowing tap water or ice water. Heat was supplied by 100 watt lamps covered with tin-foil and immersed in the water contained in the inner compartment. The lamps were connected with a mercury thermoregulator capable of regulating the temperature of the inner bath to ±.1°C. Mechanical stirrers were placed at one end of this bath.

The *Nitella* cells were contained in large beakers or jars set in the inner compartments and were illuminated by two 100 watt lights approximately 1 foot apart and suspended about 1 foot above the containers. Porcelain reflectors were used. It was found that the radiation from the lights caused the upper few inches of solution in which the cells were immersed to be several tenths of a degree higher in temperature than the body of the solution, but the increase was the same for both temperatures compared. In any case, it was evident that the temperature control was much more accurate than the possible control of other factors. In this series of experiments, the illumination was continuous and daylight was excluded.

TABLE II.

Concentrations of Br in Sap from Nitella Cells after Exposure to Bromide Solutions Kept at Different Temperatures.

No. of experiment.	Temperature.	Concentration of Br in cell sap.	Temperature coefficient (10°C.).	Concentration of Br in expressed sap.	Temperature coefficient (10°C.).	Period of exposure.
	°C.	*m.*-EQ.		*m.*-EQ.		*hrs.*
1	10	.4	3.5	.3	2.7	6
	20	1.4		.8		
2	10	2.2	2.9	1.2	2.7	25
	20	6.4		3.2		
3	10	4.3	2.9	2.7	2.6	50
	20	12.3		7.0		
4	10	4.0	2.5	1.9	2.6	52
	20	10 0		5.0		
5	10	7 1	2.3	3.6	2.4	68
	20	16.6		8.7		
6	14	12.5	2.0	6.6	2.2	72
	24	24.4		14.7		

In Experiments 1, 5, and 6 the values are averages of duplicate experiments.

Phosphate buffer solutions + .005 M KBr. Initial pH 4.8–5.1. Final pH 5.6–5.8.

Continuous illumination with two 100 watt lights, suspended approximately 1 foot above the jars containing the cells. Temperatures of bath kept within ± .1°C., except for occasional short periods when adjustments were being made.

In Table II are presented results showing the concentrations of Br found in the sap of cells which had been exposed to solutions kept at temperatures 10°C. apart, *i.e.* 14–24°C., and 10–20°C. It would have been desirable to have made measurements at numerous intervals of time for each temperature, but this would have involved the use of many large containers kept under definite temperature control, for which no facilities were available. However, while velocity constants could not be calculated, the data, taken as a whole, seem to indicate quite clearly that the temperature coefficient (between 2.0 and 3.0 for 10°C.) for the absorption of bromine under the conditions specified is of the order of magnitude generally characteristic of chemical reactions rather than of diffusion processes, and this is the main point of interest at present.

Effect of Light on Accumulation of Br.

Earlier experiments showed that the removal of Cl from dilute solutions was definitely influenced by the conditions of illumination. Several preliminary experiments also indicated that the penetration of NO_3 or of Br into the sap of *Nitella* cells was likewise accelerated under the influence of light. In the first series of the present experiments, the temperature arrangements above described were utilized. The exclusion of light, when desired, was accomplished with large beakers painted black on the outside and covered loosely over the top with black paper. The periods during which the cells were exposed to the solution containing bromide (buffer solution plus 5 milli-equivalents KBr) were relatively short, but there was a marked difference between the illuminated and unilluminated cells in respect to the concentrations of Br present in the cell sap at the end of the experiment (Table III). Except in one instance, the concentrations of Br in the samples of sap obtained from the illuminated cells were from about two to four times those in the sap from the unilluminated cells.

Subsequently, numerous additional experiments were performed over longer periods of time, without the use of the temperature baths, but with such small differences in temperature between the illuminated and unilluminated cells as to be negligible for this purpose. In every case, exposure to light strikingly increased the ability of the cells to accumulate Br. In fact, when solutions of 5 milli-equivalents KBr

TABLE III.

*Concentration of Br in Sap from Illuminated and Unilluminated Nitella Cells after Exposure to Bromide Solutions.**

No. of experiment.	Temperature.	Concentration of Br in cell sap.	Light condition.	Period of exposure.
	°C.	m.-EQ.		hrs.
1	10	2.2	In light.	25
	10	1.7	" dark.	
	20	6.4	" light.	
	20	2.6	" dark.	
2	10	4.3	" light.	50
	10	1.9	" dark.	
	20	12.3	" light.	
	20	3.3	" dark.	
3	10	4.0	" light.	52
	10	2.2	" dark.	
	20	10.0	" light.	
	20	3.2	" dark.	

* Solutions, illumination, and temperature arrangements same as described in Table II.

TABLE IV.

Concentration of Br in Sap from Nitella Cells Exposed to Bromide Solutions in Continuous Darkness, with Longer Periods of Exposure.

No. of experiment.	Medium.	Period of exposure.	Concentration of Br in cell sap.
		days	m.-EQ.
1	Tap water and 5.0 m.-Eq. KBr, in dark.	5	5.0
	Same, in daylight.	5	11.2
2	KBr, 5.0 m.-Eq. in dark.	7	5.2
	Same, in daylight and artificial light.	7	19.3
3	KBr, 5.0 m.-Eq. in dark.	5	5.0
4	KBr, 5.0 " " "	2	2.5
5	KBr, 5.0 " " "	2	1.6
	Same, in dark.	6	4.3

KBr solutions without buffer salts.

These experiments were carried out at room temperature, averaging about 20°C. At the end of the longer periods in the dark, many small cells had died, but the larger cells from which sap was obtained were turgid.

were employed, the concentration of Br in the sap of cells kept in the dark did not exceed that present in the outside solution; while the sap from illuminated cells subjected to similar solutions for equal periods of time contained much higher concentrations of Br. This was true of several different media containing bromide (Table IV). When media with concentrations of 1 milli-equivalent KBr were employed, there was considerable evidence that the concentration of Br in the sap could exceed that of the solution, even under conditions of darkness, but the quantities involved were too small to admit of certainty.

It was not found possible to keep the cells in the dark for a very extended period because of injury which was accelerated by the development of microorganisms. The question then arose whether cells kept under a normal condition of alternating periods of light and darkness could concentrate Br in the sap during the periods of darkness. It appeared that this point could be tested by alternating solutions in such a manner that some cells would have access to Br only during the periods of illumination and other cells only during the periods of darkness. Accordingly, two experiments of this type were carried out. In the first experiment, the periods were divided approximately into 12 hours of illumination and 12 hours of darkness. At the end of each period, a transfer of solutions was made, after washing the cells very thoroughly with distilled water. The result of this experiment (Table V) was that the sap from the cells having access to Br only during the periods of illumination contained Br in a concentration several times that of the external solution, but in the sap from the cells immersed in the bromide solution only during periods of darkness, the concentration of Br was not greater than that of the outside solution.

This experiment did not indicate that there was any residual effect of light but it was deemed advisable to make a further experiment over a longer total period of time, with 24 hour periods of illumination followed by 24 hour periods of darkness. The solutions were changed in the same way as before. Again a marked difference was found between the cells kept under the two conditions, but the sap from the cells placed in the bromide solutions only during periods of darkness had accumulated Br in a concentration significantly greater than that of the outside solution. The concentration in the sap was also much greater than could occur with cells kept an equal time in continuous darkness. Evidently, under suitable conditions, some effect of the illumination can be carried over to a subsequent period of darkness and influence the absorption or accumulation of substances. It cannot be stated at present whether or not this effect is concerned with a storage of available carbohydrates, although such a suggestion is a

TABLE V.

Absorption of Br in Dark by Cells Exposed to Alternating Periods of Light and Darkness.

	Concentration Br in cell sap.	Concentration Cl in cell sap.
Experiment 1.		
(Approximately 12 hr. periods of alternating light and darkness. Total duration 101 hrs.*)		
	m.-EQ.	*m.*-EQ.
A. Cells having access to KBr (concentration 5.0 m.-Eq.) only when illuminated.	18.2	102.5
B. Cells having access to KBr (concentration 5.0 m.-Eq.) only when in dark.	3.9	116.7
Experiment 2.		
(Approximately 24 hr. periods of alternating light and darkness. Total duration 10 days.)		
A. Cells having access to KBr (concentration 5.0 m.-Eq.) only when illuminated.	37.8	96.4
B. Cells having access to KBr (concentration 5.0 m.-Eq.) only when in dark.	19.0	112.5
C. Cells having access to KBr (concentration 1.0 m.-Eq.) only when illuminated.	24.9	106.0
D. Cells having access to KBr (concentration 1.0 m.-Eq.) only when in dark.	6.4	115.8

Values for concentrations in sap, averages of duplicate experiments. Solutions were buffered with phosphate, as in other experiments.

Experiment 1.—Illumination by two 300 watt lights suspended about 1 foot above jars. Temperature 24°C. ± 1.0°C.

Experiment 2.—Illumination by two 300 watt lights suspended about 1 foot above jars. In this experiment, diffused daylight supplemented the artificial illumination. Temperature 20–25°C.

* First period in light was 17 hrs. duration.

natural one. We know that certain types of cells must always be confined to the indirect use of light energy through the oxidation of carbohydrates. In the case of the *Nitella* cells, the ability to store easily available sugars seems to be very limited.

The next experiment on the effect of illumination was planned for the purpose of determining how varying the periods of illumination during each 24 hours would influence the amounts of bromine concentrated in the cell sap. In this experiment, the jars containing the *Nitella* cells were placed in a glass chamber originally designed for studying the growth of wheat plants under controlled light conditions. The chamber was illuminated by six 500 watt Mazda lights (with metallic reflectors) arranged about the sides and ends of the chamber, and approximately 3 feet distant from the jars containing the *Nitella* cells. The temperature was controlled at 23–25°C. with the aid of an air current flowing through the chamber. The light was cut off from certain cells when required by placing over the jars cylinders of heavy brown paper with loose coverings for the top. This arrangement shut out practically, but not absolutely all the light. The illuminated solutions had a slightly higher temperature, about 1°C., than the others, but the effects of varying the periods of illumination were so great that this factor was negligible in comparison.

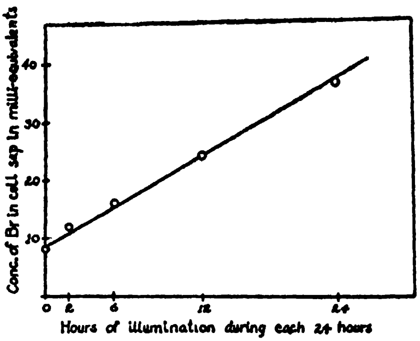

FIG. 1. Relation between number of hours of illumination and accumulation of Br in sap of *Nitella* cells. During periods of exposure, cells were illuminated by six 500 watt Mazda lights. (See text.)

Referring to Fig. 1, it will be observed that there was a striking increase in the concentration of Br in the cell sap with each increased period of illumination. The greatest concentration was found in the sap from cells exposed to light continuously for 3 days. The concentrations of Br do not vary in direct proportion to the periods of illumination, but an approximately straight line is formed by plotting the hours of light against Br concentrations. The total number of points is not sufficiently great to warrant an attempt at a mathematical analysis, but there can be no doubt of the influence of the length of the periods of illumination. This conclusion is in accord with our previous observations on the removal of Cl from dilute solutions.

With regard to the possible relations existing between quality or intensity of light and the accumulation of Br, it will be necessary to make additional experiments. For the present, it may be noted that, in general, the most rapid accumulation occurred where the intensity of light was greatest. One experiment was carried out for the specific purpose of comparing two intensities of light, maintaining the temperature the same (approximately 20°C.) in both cases within .2 of a degree. It was found that doubling the light (100 and 200 watts) increased the absorption 30 per cent as an average of duplicate experiments which were in close agreement. In another test, the results indicated that yellow light (potassium chromate solution filter), was at least as efficient as the white light from Mazda lamps. Blue light (alkaline copper solution filter) was less effective, but this observation has no necessary significance since the total energy values of the different lights are not known.

The Effect of Toxic Agents.

The ability of *Nitella* cells to concentrate various substances in their cell sap is apparently bound up with the processes of growth or metabolism. Toxic agents might, therefore, be expected to interfere with such a concentrating action. We have made several experiments which seem to support this assumption (Table VI). Different toxic substances were added to the bromide solutions and the concentration of Br in the cell sap determined as in the other experiments. In some cases, many small cells were killed by the toxic substances, but the samples of sap used for the analysis were, of course, obtained from those large cells which still remained turgid at the end of the period of exposure. It is reasonable to suppose, however, that these cells also suffered some injury. Prolongation of the treatments would have resulted in the death of all the cells.

The injury has evidently caused, or been accompanied by, a decreased ability on the part of the cells to concentrate bromine in the cell sap. Tröndle,[6] by indirect methods, has reached a somewhat similar conclusion with the use of various salts. While, in the case of anesthetics, the electrical resistance of certain plant cells may be increased over a limited period of time,[7] any marked injury generally involves increased permeability, as, for example, in the experiments

TABLE VI.

Effect of Toxic Agents on Concentration of Br in Cell Sap.

No. of experiment.	Treatment.	Concentration of Br.	Period of exposure.
1	None.	28.8	3 days continuous illumination.
	1 p.p.m. KCN.	29.0	
	10 " "	18.2	
2	None.	19.7	5 days daylight.
	Chloroform.	12.5	
3	None.	13.5	4 " "
	10 p.p.m. KCN.	14.1	
	20 " "	8.7	
4	None.	22.6	4 " "
	Chloroform.	9.4	
	Ether.	14.7	
	20 p.p.m. KCN.	12.5	
	Thymol excess.	12.5	

Phosphate buffer solutions containing 5.0 milli-equivalents KBr. 1 cc. of ether or chloroform added to 3 liters of solution. Treatment with ether repeated several times. Additional chloroform added once in Experiment 4. In Experiment 4, results are averages of duplicate experiments, except for last treatment. The differences between duplicates were much smaller than those between the control and the treated cells.

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[6] Tröndle, A., *Biochem. Z.*, 1920, cxii, 259.

[7] Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Monographs on experimental biology, J. B. Lippincott Company, Philadelphia and London, 1922.

of Osterhout[8] and Brooks[9] on *Nitella* cells. In our view, such increased permeability may be accompanied by a decreased ability to concentrate substances in the sap. This important distinction will be referred to again in the later discussion.

The Exchange of Substances between Cell Sap and Culture Solution.

Before giving further consideration to the accumulation of Br in the cell sap, it is necessary to inquire into the possibility of an exchange of ions between the cell sap and the culture medium. Previous work had shown that the contents of the sap did not appear to diffuse out unless the cells were injured. When masses of healthy and uncon-

TABLE VII.

Exchange of Br and Cl between Cell Sap and Culture Medium.

Composition of original solution.	Found in solution after contact with cells.		Period of exposure.
	Br	Cl	
Experiment 1.			
	m.-EQ.	*m.-EQ.*	*days*
Phosphate buffer + KBr 5.0 m.-Eq.	3.85	.68	7
" " + KCl 5.0 "	.44*	3.66	
" " + KBr 5.0 "	2.44	1.38	43
" " + KCl 5.0 "	.90*	3.24	
Experiment 2.			
Phosphate buffer + KBr 5.0 m.-Eq.	2.40	1.24	21

Cells kept at room temperature in daylight. The proportion of cells to solution was approximately 30 gm. of the former to 600 cc. of the latter.

The masses of cells were composed chiefly of small, very green and turgid cells, practically all in healthy condition, judged by appearance. No evidences of injury developed during the experiment. Similar cells kept in the phosphate solutions without Cl or Br did not lose these elements to the solution sufficiently to give a definite test with $AgNO_3$.

* The cells used in these tests had previously been exposed for several months to solutions containing .002 M KBr.

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[8] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, v, 709.

[9] Brooks, M. M., *J. Gen. Physiol.*, 1921-22, iv, 347.

taminated cells were immersed in a phosphate buffer solution, for example, the test for Cl in the solution was negative or only indicated a very slight trace, even after the lapse of several weeks. The same observation has been made with solutions containing sulfate or nitrate. But under exactly similar conditions, except with the substitution of bromide, very appreciable quantities of Cl were found in the external solution after from 1 to 6 weeks (Table VII), although in this time the cells presented no evidence of injury, judged by their appearance. In the course of a month, considerable new growth had occurred. The amounts of Cl lost from the cells were of such magnitude that they would have been followed by obvious signs of injury or death of the cells, had the loss occurred from cells exposed to solutions not containing bromide. Furthermore, when cells which had previously accumulated a high concentration of Br in their sap were placed in solutions containing chloride, Br entered the solution, but no appreciable amount was lost to similar solutions not containing chloride. For these reasons, it is very difficult to ascribe such exchanges to cell injury. If injury were present, it would seem that it must have been too slow to account for the effects noted. Another point to be emphasized is that Cl may be lost from cells at the same time Br is being concentrated in the sap. The studies on the Br and Cl content of the sap itself will now be described.

Accumulation of Br over Various Time Intervals.

In these experiments, Br determinations on the sap were made as before. Cl was determined on the same samples and the changes in its concentration were estimated by obtaining the difference between the concentration of Cl in the sap from untreated cells and in that from similar cells after exposure to the solutions under investigation. In many cases, the differences were comparatively large and since each sample was representative of a great many cells, the values for the increases or decreases of Cl concentration determined in this way are undoubtedly significant of loss or gain of Cl by the cell sap.

We now desire to direct attention to Fig. 2 in which are plotted the changes in Br and Cl concentrations of cell sap, which occurred over a period of 40 days. The cells were kept continuously illuminated and while the temperature could only be controlled very roughly, the average temperatures for each time interval were sufficiently alike so that no important fluctuations in the general trend of the curves is noted. It will be observed, first, that the accumulation of Br takes place in a very gradual manner and that 40 days elapsed before a condition of apparent equilibrium was attained. This slow accumulation of Br in the cell sap was very definitely accompanied by slow loss of Cl until at the end of 40 days, the equivalents of Cl and Br present in the sap were almost equal. At this time, the masses of cells all appeared to be in a healthy condition.

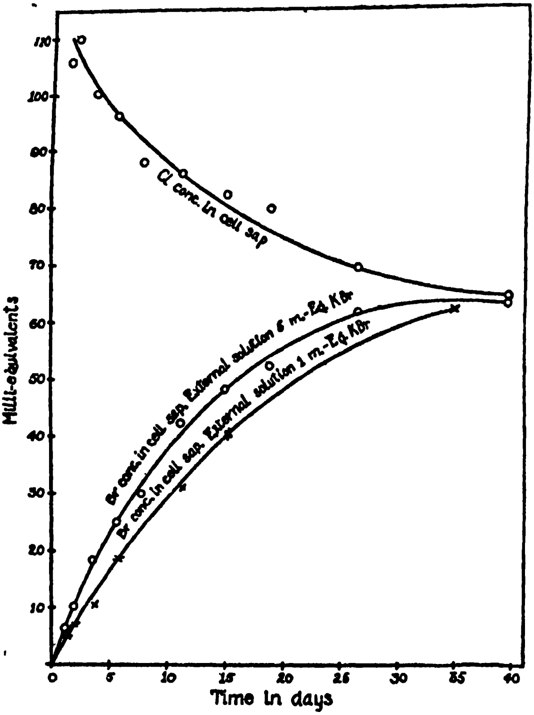

FIG. 2. Rate of accumulation of Br and loss of Cl over period of 40 days. Continuous illumination. Diffused daylight supplemented by artificial illumination by two 300 watt Mazda lights.

Values averages of duplicate or triplicate experiments, agreeing usually within 10 per cent. Error of 25 per cent may occur with very small concentrations of Br.

While a loss of Cl took place, it is especially to be noted that the total halogen content of the sap was decidedly increased. In other words, the equivalents of Br accumulated exceeded those of Cl lost from the cells.

In this experiment, comparison was also made of solutions containing 5 milli-equivalents and 1 milli-equivalent of KBr. The two curves representing the increases in the concentrations of Br in the cell sap are not widely separated and at the conclusion of the experiment, the concentrations in the sap of the cells kept in the solution of 1 milli-equivalent KBr and in that of 5 milli-equivalents KBr were almost the same. Evidently, there is no equality in the ratios of internal to external Br concentration with different concentrations of bromide present in the culture solutions. Further data pertaining to this point will be presented in a later discussion.

Recently Irwin[10] has subjected her data on the accumulation of a dye by *Nitella* cells to a mathematical analysis using the formula $K = \frac{1}{t} \log \frac{a}{a-x}$, in which K is a constant, t time, a concentration of dye in sap at equilibrium, and x concentration of dye at time t. It was thought that some interest might attach to the application of a similar formula to the data plotted in Fig. 2. (In this case, a and x refer to Br concentrations.) With the solution containing 5 milli-equivalents of KBr, a fairly good agreement is found between observed and calculated values of x, having in mind the difficulties of experimentation (Table VIII). The number of observations on the solution of 1 milli-equivalent KBr concentration was too small for the purpose but a lower value of K for this solution is indicated. With regard to the rate of loss of Cl, a lower value of K was obtained than for the accumulation of Br.

While the formula applied by Irwin to the accumulation of dye may fit approximately the data for the accumulation of Br, it does not, of course, follow that the two processes are necessarily similar. There is a very striking difference in the time required for the attainment of an equilibrium condition. In the case of the dye, this condition was reached in a few hours, while for Br 30 or 40 days was necessary, even under favorable light and temperature conditions. The ratio between the concentration of dye in the sap and in the external solution was found to be constant in the range studied, but such constancy of ratios is far from applying to the accumulation of Br. It was concluded that the dye very possibly combines with some organic constituent of the sap. There is no evidence that the accumulation of Br can be explained on the basis of organic combinations in the vacuole, although it may well be true that intermediate processes involve such combinations with some constituent of the protoplasm.

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[10] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

TABLE VIII.

Accumulation of Br in Cell Sap of Nitella.

(Fig. 2.)

Calculated according to formula used by Irwin for accumulation of dyes.

$$K = \frac{1}{t} \log \frac{a}{a - x}$$

k = constant, a, concentration of Br at equilibrium.
x = concentration of Br at time t.
a = 64.6.
Br concentrations in milli-equivalents.

Time.	K	x observed.	x calculated. K = .037
days			
1.15	.036	6.1	6.3
2	.037	10.1	10.1
4	.037	18.6	18.7
6	.037	25.8	25.8
8	.034	30.4	31.9
12	.039	42.8	41.4
16	.038	48.4	48.1
20	.038	53.4	52.9
28	.038	63.2	58.7
40	.036	64.6	62.5
Average...........	.037		

Culture solution phosphate buffer + KBr, 5 milli-equivalents, same as used in other experiments.

Other Reciprocal Relations of Br and Cl.

In connection with the displacement of Cl by Br, the question arose whether cells with Cl content initially higher than that of normal sap could accumulate Br with the same rapidity as normal cells (*i.e.* cells kept in tap water). Several experiments were made with this point in mind. During an initial period of 10 to 14 days, certain lots of cells were exposed to a buffer solution without Cl and others to a similar solution to which 5 milli-equivalents of KCl had been added. At the end of this initial period, the cells (A) which had been in the solution without Cl were transferred to a solution containing 5 milli-equivalents KBr. A portion of the cells (B) which had been kept in the chloride solution were also placed in a solution containing KBr. Another portion of the cells (C) from the chloride solution was transferred to a solution without Cl or Br. The second period of the experiment was from 5 to 9 days in length. In each experiment cells were maintained under the same light and temperature conditions. At the end of the final periods, samples of sap were obtained from the various lots of cells and determinations of Cl and Br were made (Table IX).

Attention is first called to the fact that the Cl content of the cell sap (C) could be increased from 30 to 40 per cent under the conditions described. These and other experiments suggest, however, that the accumulation of additional Cl is less rapid than the accumulation of Br. The second important observation is that the cells (B) which had previously had their Cl concentration increased accumulated a much smaller amount of Br than did the cells (A) which had a normal Cl content in the sap at the time they were placed in the bromide solution. In the latter cells, a considerable concentration of Br was attained without anything like an equivalent displacement of Cl, but the accumulation of Br by the cells with the initially higher Cl concentration was at the expense of a nearly equivalent displacement of Cl. The cells behaved as though the total halogen content could be increased to a certain point, after which accumulation of halogens occurred only as a result of exchange or displacement. The consistent data obtained in three independent experiments under different conditions, seem to warrant these statements.

Conductivity Data.

If the total electrolyte content of the cell sap can be increased, we should be able to show that increases in conductivity also occur. In order to determine the extent of such changes, a special conductivity

TABLE IX.

Retarding Effect of Preliminary Accumulation of Cl on Subsequent Accumulation of Br.

Culture solutions (a).	Concentration of Cl in cell sap.	Concentration of Br in cell sap.	Light and temperature conditions.
Experiment 1.			
	m.-EQ.	*m.*-EQ.	
A. Period 1, no Cl or Br added. " 2, KBr, 5 m.-Eq.	97.2	32.0	Period 1, 14 days; Period 2, 9 days. Continuous illumination (b).
B. " 1, KCl, 5 " " 2, KBr, 5 "	122.0	13.8	Same conditions.
C. " 1, KCl, 5 " " 2, no Cl or Br added.	137.5	0	" "
Experiment 2.			
A. Period 1, no Cl or Br added. " 2, KBr, 5 m.-Eq.	103.0	40.1	Period 1, 10 days; Period 2, 5 days. Continuous illumination (c).
B. " 1, KCl, 5 " " 2, KBr, 5 "	117.5	22.7	Same conditions.
C. " 1, KCl, 5 " " 2, no Cl or Br added.	141.0	0	" "
Experiment 3.			
A. Period 1, no Cl or Br added. " 2, KBr, 5 m.-Eq.	101.5	22.4	Period 1, 10 days; Period 2, 6 days. Continuous illumination (d).
B. " 1, KCl, 5 " " 2, KBr, 5 "	129.5	10.9	Same conditions.
C. " 1, KCl, 5 " " 2, no Cl or Br added.	137.2	0	" "

All experiments carried out in duplicate, with agreement within 10 per cent of total value, except in one case. Average values given.

(a) Phosphate buffer solutions + 5 milli-equivalents KBr or KCl, as indicated. pH approximately 5.4

(b) Illumination by two 300 watt lamps placed about 2 feet above jars, in addition to diffused daylight. Temperature average 20–22°C.

(c) 3 days illumination as in b, remainder of first period and all of second period illumination by six 500 watt lamps in light chamber. Temperature average, 18–20°C.

(d) First period in light chamber as in c; second period illumination as in b.

The Cl content of the untreated cells was as follows:

Experiment 1–101 milli-equivalents.
" 2–103 "
" 3–108 "

TABLE X.

Comparison of Conductivities of Normal Cell Sap and Sap from Cells Exposed to Solutions Containing KBr.

(Phosphate buffer solutions with 5 milli-equivalents KBr.)

Collection of cells.	Conditions of exposure.	Concentration of Cl in cell sap.	Concentration of Br in cell sap.	Specific resistance of cell sap.	Percentage decrease in resistance.
		m.-EQ.	*m.*-EQ.	*ohms*	
B	6 hrs. continuous illumination (1).	98.6	1.4	74.7	
B	6 days, same conditions (1).	95.4	37.8	59.4	21
B	9 " " " (1). (KBr, 20 m.-Eq.)	97.0	53.1	56.2	25
C	No treatment.	105.5	0	77.2	
C	10 days (7 days intermittent and 3 days continuous illumination).	90.9	49.6	62.3	19
C	17 days daylight (3).	83.4	33.4	69.2	10
C	38 " continuous illumination (4).	69.1	64.8	58.9	24
D	No treatment.	101.1	0	74.3	
D	10 days (4 days daylight and 6 days continuous illumination) (5).	Not determined.	64.5	51.2	31
D	9 days continuous illumination (6).	98.0	31.4	59.0	21
D	19 days daylight (7).	88.0	51.4	59.6	20
E	No treatment.	103.0	0	76.4	
E	16 days continuous illumination (8).	82.2	48.4	61.8	19
E	18 days continuous illumination (9).	80.0	56.6	54.8	28
E	20 days continuous illumination (10).	81.0	53.4	60.1	21
E	5 days continuous illumination (11).	105.0	41.0	54.9	28

(1) Continuous illumination by two 300 watt lamps, suspended about 1 foot above jars. Temperature 22–26.5°C.

(2) 7 days, diffused daylight, supplemented by two 300 watt lamps during periods of 7–8 hrs.; 3 days light on at night also. Temperature 20–25°C.

(3) Diffused daylight only. Temperature average approximately, 20°C.

(4) Diffused daylight in addition to continuous illumination with two 300 watt lamps, 2 feet above jars. Average temperature 20–22°C.

(5) Diffused daylight and illumination during 6 nights by two 300 watt lamps, 1 foot above jars.

(6) Same conditions as in (4).

(7) Diffused daylight only. Room temperature.

(8) Same conditions as in (4).

(9) Illumination by six 500 watt lights, light chamber. Average temperature approximately 20°C.

(10) Same conditions as in (4).

(11) " " " " (9).

cell was constructed for use with slightly less than 1 cc. of cell sap. Conductivity determinations were made in various experiments and certain typical data are given in Table X. In every case in which Br accumulated to any great extent a significant increase of conductivity occurred. These increases varied from 10 to 31 per cent, based on the values for sap from untreated cells. In certain instances, the increases of conductivity were of a magnitude very similar to those which would be obtained by adding to normal sap an equivalent amount of KBr, but in other cases, the increase is decidedly less than would correspond to this condition, as might be expected, considering that Cl ions have been displaced. Unpublished data indicate that an exchange of bases, especially sodium for potassium, may also occur under some circumstances. If these exchanges are taken into consideration, there is nothing in the conductivity data to suggest that an appreciable amount of Br has been organically combined. Other reasons for this view, previously discussed in connection with the chloride content of the sap also apply to the bromide content.

While the quantitative responses of different lots of cells collected at different times of the year are not exactly the same, there are one or two general relations which are strongly suggested by the available data. In several experiments, a large accumulation of Br took place with little or no loss of Cl. In these cases, the light and temperature conditions were very favorable to the accumulation and a high concentration of Br was attained in the cell sap in a relatively short period of time. In comparing two specific experiments conducted under different conditions, we note that in 6 days, with a high intensity of continuous illumination, 37.8 milli-equivalents of Br were accumulated with a loss of 3.2 milli-equivalents of Cl. In the other experiment, with diffused daylight alone, and with a lower temperature, 17 days were required to accumulate 33.4 milli-equivalents of Br and the loss of Cl was 22.1 milli-equivalents. While these relations can only be suggested at the present time, it is evident at least that a gain of Br and loss of Cl do not proceed necessarily at the same rate and that light, temperature, and time are important factors in all processes.

It cannot be proved, of course, that exchanges of ions are, strictly speaking, involved. Since it is also possible that cations may be displaced, as well as anions, it could be assumed, for example, that KBr or K and Br ions entered the cell and that NaCl or Na and Cl ions left the cell. However, the course of events is certainly more complex than this statement would imply.

There are various other data which can be discussed later when certain additional experiments have been made, but a few preliminary observations may be made now incidentally. With regard to the effects of hydrogen ion concentration on the accumulation of Br, our present results do not show that these effects are the same as those obtained in the studies on nitrate, which accumulates much more slowly than Br. The influence of certain other anions on the accumulation of Br is striking, and in single salt solutions, the nature of the cation plays a very important rôle.

DISCUSSION.

In most of the investigations on the absorption of substances by plant cells, the latter are assigned a more or less passive rôle and the principal conclusions are concerned with explanations of alterations of permeability occurring under diverse conditions. This, however, can be only a partial view of the situation. From the point of view of the growth and nutrition of the plant, it is highly essential to emphasize the ability of the cell to concentrate, or, in the sense of the word as recently employed by Osterhout, to accumulate substances in its interior. All the evidence now available shows that it is possible for certain inorganic elements to be taken out of a dilute solution and stored in a solution of much higher concentration inside the cell. It is true that such processes may take place relatively slowly, but, nevertheless, often at an appreciable rate, as we have shown with regard to Cl and Br. In most physiological experiments which have been reported, no adequate idea of the intake of inorganic elements by plant cells has been obtained because the time intervals employed were too short.

The ability of living cells to concentrate substances has, of course, been recognized by various writers and in this connection, it seems worth while to quote the following from a recent book by Lillie:[11]

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[11] Lillie, R. S., Protoplasmic action and nervous action, University of Chicago Press, Chicago, 1923.

> "But since these compounds (crystalloidal compounds) do, in fact, gain entrance to the cell, at least at certain times, it is clear that the problem of cell permeability is not a simple one. Apparently, we must conclude that the entrance or exit of substances by simple diffusion is, in most cases, a different phenomenon from their entrance, or exit, under physiological conditions. The processes of absorption and secretion are, in fact, special activities, requiring the performance of work by the cell. The distinction between a passive or purely physical permeability and an active or physiological permeability thus seems to be a necessary one."

In view of the importance of such distinctions, it is regrettable that the term permeability has been used to describe so many types of phenomena. The general adoption of the term "accumulation" to designate certain processes, as in the recent usage of Osterhout, might serve to clarify discussion.

The experimental data presented in this article seem to offer definite evidence that light is an essential factor in the accumulation of Br or Cl by *Nitella* cells. Blackman and Paine, Tröndle, Lepeschkin[12] and several others, have described the effects of light on permeability, but in these investigations, the methods were indirect and permeability, as such, was the chief point of interest. Results such as we have obtained cannot be explained by the statement that light simply increases cell permeability. Light obviously contributes energy to a system, and it would seem necessary to assume that this energy, which, under appropriate conditions can be stored, may be utilized to bring about a movement of solutes from a region of low concentration to one of higher concentration.

In our earlier work, which has been confirmed, we found that Cl did not diffuse out of uninjured cells into the various types of solution which were tried, and it was questioned whether such outward movement of Cl could take place in the absence of injury. It now seems certain that an exchange of Br for Cl may take place (or its equivalent), and present information would not point to injury as the primary cause of the exchange, which seems to occur to an appreciable extent only with elements which can be absorbed or accumulated by the cell with relative rapidity. Notwithstanding the existence of such exchange phenomena, Br and Cl can both accumulate in the cell in concentrations much higher than those present in the external solution, so that our views with regard to concentration gradients are not altered, but only extended.

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[12] See review by Stiles, W., Permeability, Wheldon and Wesley, London, 1924.

It is now of interest to consider the suggestions recently advanced by Osterhout[13,14] to the effect that ions may not be able to penetrate living protoplasm, but that penetration is confined to undissociated molecules. We have been accustomed to assuming, in dealing with solutions of strong electrolytes such as those used in the experiments on *Nitella*, that the interpretation of the results could best be made in terms of ions. The dilute character of the solutions, existence of exchange phenomena apparently involving ions, the influence of one ion on the absorption of another, are some of the reasons which would seem to favor an interpretation in terms of ions, as far as the particular phenomena we have been investigating are concerned.

It is very possible the experiments carried out by Osterhout on certain chemical systems are not inconsistent with an hypothesis of ionic penetration as supplied to other systems. In his studies on the absorption of H_2S and CO_2 by *Valonia*, evidence was found that the equilibria between the sap and the sea water medium could be explained most logically on the basis that ions could not penetrate the cell. The question then arises whether the failure of the ions of the H_2S and CO_2 systems to penetrate necessarily implied that Br and Cl ions cannot do so. While the question is not now capable of a definite answer, several points of difference between the experiments with H_2S and CO_2, and the experiments with Br and Cl should be noted. In the first place, the time periods involved are very much longer in the latter case, so that opportunity would be afforded for the very slow entrance of ions. In the case of the studies on H_2S, with *Valonia* it was not found that a higher concentration of sulfide was attained inside the cell than outside, while *Nitella* cells show a marked ability to concentrate Br or Cl in the sap. Then, in the H_2S and CO_2 systems, gaseous components were present, and it is quite possible that these were involved in such a way as to make it very uncertain whether any direct comparison can be made between the results

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[13] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

[14] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131.

obtained with Cl and Br, and those with CO_2 and H_2S. Still another point of difference is found in the temperature coefficient. Osterhout found a low coefficient for the entrance of H_2S, while in our experiments the coefficient for the accumulation of Br is of the order of magnitude of a chemical process.

Looking at the whole question from another point of view, it is not certain that we are now in a position to decide whether we are dealing with undissociated molecules or with ions, for the reason that intermediate chemical compounds may be formed, the chemical nature of which is unknown. If combinations of this sort are, in fact, involved, information concerning their character and the energy relations of their formation or dissociation would appear to be indispensable to an understanding of the mechanism of penetration of those substances which can accumulate in the cell sap of plants. It will also be necessary to determine whether these phenomena are concerned at all with electrical potential differences. In any case, the energy relations implied in the ability of living plant cells to concentrate substances require consideration irrespective of the mechanism of accumulation.

CONCLUSIONS.

1. By the use of a special analytical technique it has been possible to study the accumulation of halogens in the cell sap of *Nitella*.

2. From a dilute solution, Br may be accumulated in the sap in a concentration much greater than that of the external solution. The conductivity of the sap may be markedly increased by such accumulation. The process is a slow one so that a month or more may be required to approach equilibrium.

3. Cl may be lost from the cell as a result of the accumulation of Br and *vice versa.* Other reciprocal relations between Cl and Br are indicated.

4. At equilibrium practically as much Br accumulated in the sap with an external solution containing 1 milli-equivalent of Br as with one containing 5 milli-equivalents.

5. Light energy was indispensable to the accumulation of Br. The temperature coefficient was characteristic of a chemical process.

THE EFFECT OF THE pH ON THE GERMICIDAL ACTION OF SOAPS.

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That soaps possess a marked germicidal action has been known since the time of Robert Koch (1881). This valuable property finds every-day application in the sterilization of the skin, dishes, and soiled clothing. Soaps are furthermore of interest to the bacteriologist because they, or substances similar to them, very probably have something to do with the body's defence against infections (see Flexner's introduction to the paper of Lamar, 1911, *a*).

Solutions of soaps having 12 or more carbon atoms in the molecule are alkaline in reaction, because of hydrolysis. The longer the chain of carbon atoms, the greater is the hydrolysis and the more alkaline is the solution. Most investigators of the germicidal action of soaps are agreed that this alkaline reaction favors the destructive effect on the bacteria. Reichenbach (1908), working with *B. coli* as test organism, found that the more strongly hydrolyzed and therefore more alkaline soaps were most germicidal; addition of alkali increased the germicidal titer. Lamar (1911, *b*) found sodium oleate to be more hemolytic than oleic acid; this difference he ascribed to the lower solubility of the acid. Nichols (1919–20) found that the soaps commonly used in washing dishes (the resinates, stearates, and palmitates) were precipitated and lost their germicidal action when the reaction was brought to pH 7.0 or more acid. Further than this, there seem to be no observations recorded in the literature on the effect of the pH on the germicidal action of the soaps.

EXPERIMENTAL.

Soaps of the following normal fatty acids were investigated: butyric, caproic, caprylic, capric, undecylic, lauric, tridecylic, myristic, pentadecylic, palmitic, stearic, oleic, and ricinoleic. The butyric, caproic, and oleic acids were Kahlbaum's; the remainder were obtained from the Eastman Kodak Company. The soaps were prepared by adding the theoretical quantity of fatty acid to N/5 KOH. Potassium soaps were chosen because of their greater solubility. Walker (1924) has shown that the sodium and potassium soaps have practically the same germicidal value. Before each experiment, serial dilutions of the soaps required were made with sterile distilled water.

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TABLE I.

Composition of Buffer Mixtures.

pH	M/5 KH_2PO_4	N/5 KOH	N/10 glycine.	N/10 KOH
	cc.	*cc.*	*cc.*	*cc.*
5.0	9.90	0.10	10.0	0
5.5	9.71	0.29	10.0	0
6.0	8.97	1.03	10.0	0
6.5	7.67	2.33	10.0	0
7.0	6.28	3.72	10.0	0
7.5	5.50	4.50	10.0	0
8.0	5.17	4.83	9.75	0.25
8.5	5.10	4.90	9.0	1.0
9.0	5.10	4.90	8.0	2.0
9.5	5.10	4.90	6.5	3.5
10.0	5.10	4.90	5.5	4.5

TABLE II.

Composition of Lactic Acid-Potassium Lactate Buffer Mixtures.

pH	N/10 potassium lactate.	N/1 lactic acid.
	cc.	*cc.*
5.0	10.0	0.0625
4.7	10.0	0.125
4.4	10.0	0.25
4.1	10.0	0.50
3.8	10.0	1.00

The pH of the soap solutions was controlled by the addition of buffer mixtures, whose composition is given in Tables I and II.

The composition of the phosphate and glycine buffers was calculated from the data given in Clark (1922). The pH of the glycine-

KOH mixtures is for 37°C. The phosphate mixtures were made up in larger quantities; the glycine-KOH mixtures were made up fresh for each experiment and added to the phosphate.

Five different species of test organisms were used. These were:

1. *Streptococcus pyogenes.* The strain used was the "H" strain first described by Gay and Stone (1920); its extremely high virulence for rabbits has been maintained by constant animal passage. This strain is hemolytic; it grows vigorously and diffusely in infusion broth.
2. *B. diphtheriæ.* The "Park-Williams No. 8" was the strain used.
3. *Staphylococcus aureus.* An old laboratory stock culture was employed.
4. *B. typhosus.* The "Pfeiffer" strain was used.
5. *Vibrio choleræ.* The strain used was an old stock culture of unknown origin.

The germicidal tests were performed in the following manner:

The required buffer solutions were sterilized; when cool, they were inoculated with the test organisms and well mixed. Then 0.5 cc. quantities were pipetted into series of small sterile test-tubes. The inocula were such that each 0.5 cc. of the buffer fluid contained 0.04 cc. of an 18 to 24 hour broth culture of *Streptococcus pyogenes*, *B. diphtheriæ*, or *V. choleræ;* or 0.02 cc. of *Staphylococcus aureus* or *B. typhosus*. Then 0.5 cc. quantities of the soap solutions (serial dilutions of which were prepared in advance) were added to each tube. Finally each tube was gently rotated while in a slanting position and placed in the water bath at 37°C. At the end of 30 minutes, 2 hours, and 18 hours, a 4 mm. loopful from each tube was subcultured on plates of blood agar (*Streptococcus*, *B. diphtheriæ*, and *V. choleræ*) or plain agar (*Staphylococcus* and *B. typhosus*). In case the tube contained a precipitate of insoluble fatty acid, the precipitate was well stirred with the platinum loop on subculture. The plates were incubated at 37°C. for 48 hours and read. In several experiments duplicate subcultures were made: the first set on agar plates, the second set in tubes of infusion broth. No essential differences were observed.

The soap solutions added were in certain instances strongly alkaline. The question thus arises, how adequate are these buffer mixtures to resist change of pH on the addition of this alkali? The alkaline end of the series—from pH 8.0 on—is very well buffered against weak alkalies such as the soaps; the acid end, on the other hand, is but poorly buffered against any but very dilute solutions. It was found necessary to test the final pH of the solutions with indicators to determine where any serious shifting of pH had occurred. The less hydrolyzed soaps gave very little trouble. Those with fewer than 10 carbon atoms could easily be adjusted with acid before being added to the buffer mixtures. The potassium laurate, in concentrations of N/160 or less, caused no serious shifting of pH except in the most acid of the phosphate mixtures. The same was true of the soaps of higher molecular weight when the concentration was N/320 or less. When any shift in the pH of a significant tube occurred, the observed pH (colorimetric) was recorded. As a matter of fact, corrections of this nature were not often required.

TABLE III.

	Acid limit of tolerance.		Alkaline limit of tolerance.	
	2 hrs.	18 hrs.	2 hrs.	18 hrs.
	pH	*pH*	*pH*	*pH*
Streptococcus	5.5	6.0	9.5	9.0
B. diphtheriæ	4.4	4.7	10.5	10.0
V. choleræ	5.5	6.0	9.0	8.5
Staphylococcus	3.8	4.4	10.5	10.0
B. typhosus	4.4	5.0	10.0	9.0

The pH values given indicate the most acid or alkaline tube that gave growth on subculture. The tests were conducted in the same manner as the germicidal tests with soap, except that distilled water instead of soap solution was added to the buffer fluids.

Inasmuch as an excess of either acid or alkali is destructive to bacteria, repeated tests were made to determine the acid and alkaline limits of pH tolerated by the five organisms here studied. The results are given in Table III.

The results of the germicidal experiments with soaps are given in Figs. 1 to 7. Attention must be called to the fact that the germicidal titers given in these figures refer, not to the amount of soap or fatty acid in solution, but to the total amount added, irrespective of whether a precipitate formed or not Thus, when N/10 potassium stearate was brought to pH 6.0, practically all of it was precipitated out. When such a mixture was found to be without germicidal properties, it was recorded as having a titer of <N/10. Again, when N/640 potassium myristate was brought to pH 6.0 (as in Fig. 1) a precipitate formed, and the actual concentration of myristate in solution became much less, yet it was recorded as N/640. This procedure was adopted for two reasons: first the difficulty of estimating the amount of fatty acid actually in solution; and second, the fact that the substances in solution are in equilibrium with the precipitate, so that the latter cannot be regarded as wholly inert.

A study of the experimental data brings out the following facts.

1. Potassium butyrate is non-germicidal in a concentration of N/10 at all pH values within the limits given in Table III.

2. The lower members of the saturated series of soaps are most germicidal in an acid reaction. The most striking example of this was found in the case of potassium caprate and *Staphylococcus:* the titer here was 1000 times as great at pH 4.4 to 4.7 as at pH 9.0 to 10.0.

3. The curves for the higher members of the saturated series are the reverse of those for the lower members, showing greater germicidal action when the pH is alkaline. Certain soaps (such as the myristate and the palmitate with *Streptococcus*) are intermediate in their behavior, showing a definite minimum in their activity near the neutral point.

4. With increasing molecular weight of the soap, the germicidal titer increases to a maximum and then diminishes. The point at which this inflection occurs varies with the pH and the organisms (Figs. 5 and 6). Thus, with *B. typhosus* at pH 5.5, the titer rises from caproic to capric acid, and then falls off very rapidly through undecylic acid to lauric acid, where no germicidal action could be demonstrated. With the other four organisms tested, the maximum for the acid range was reached at lauric and tridecylic acids, with a somewhat slower falling off of the titer with increasing molecular weight. In the alkaline pH range, the germicidal action toward all five organ-

isms increases with the molecular weight to the palmitate and then diminishes.

5. The oleate and ricinoleate were not tested against all five organisms. Fig. 7 shows that the oleate with *Streptococcus* and the ricinoleate with *B. diphtheriæ* are far more effective in acid than in neutral or alkaline ranges. The oleate with *B. diphtheriæ* gave a curve quite unlike any other that was obtained, with maximal germicidal action at pH 6.5 and 7.0. The oleate showed no action on *Staphylococcus* and very little on *B. typhosus,* and then only at the most alkaline reactions. This confirms the observations of Reichenbach (1908) and Walker (1924).

From this data it is clear that the soaps have a germicidal power that varies with their structure and the species of germ acted upon; and that the corresponding fatty acid may likewise be germicidal, often far more than the soap.

The question arises, what is the cause of the sudden drop in the curves (Figs. 5 and 6) after a maximum has been reached? Why are the curves in Figs. 1 to 4 reversed as we pass from soaps of lower to higher molecular weight?

One reason for this is the diminishing solubility of the soap or fatty acid as the molecular weight increases. Thus, the most concentrated solution of potassium laurate that remains clear at pH 6.0 is N/5120. More concentrated solutions contain insoluble matter in suspension. It is impossible to determine whether N/160 dissolved laurate or palmitate would kill the typhoid bacillus at pH 6.0, because that amount cannot be obtained in solution.

There is also evidence to show that the germicidal power of the fatty acids also actually diminishes with increasing molecular weight, after a maximum has been reached. It is suggested that this may be due to diminished solubility of the germicide in the bacterial protoplasm. In the case of *B. typhosus* (Fig. 6), the sudden drop from capric to undecylic acid can be explained only by a diminution in germicidal power.

A comparison of the germicidal titers for the three different time intervals gave some rather interesting results. The following combinations ran practically the same titers for the 30 minute, 2 hour, and 18 hour periods: *Staphylococcus* with soaps of 12 or fewer carbon atoms; *B. typhosus*, *B. diphtheriæ*, and *V. choleræ* with 11 or fewer carbon atoms. With soaps of higher molecular weight, the titers were increased by lengthening the incubation period. This increase, however, was not always uniform over the whole pH range. Thus, for the 18 hour period, the curve for the myristate and *Staphylococcus* (Fig. 1) was flattened out without being raised at the ends, while the palmitate and stearate curves were shifted to the left without any change in their shape. On the other hand, the myristate curve for *V. choleræ* (Fig. 3) instead of being flattened, was strongly raised at both ends when the incubation period was increased to 18 hours.

The tests described were carried out in a salt concentration of N/20. A detailed study of the effect of salt on the germicidal action of soaps was not undertaken, but a few experiments were made which indicated that the addition of salts increases the germicidal action. One such experiment is shown in Table IV.

TABLE IV.

The Effect of the Salt Concentration on the Germicidal Titer of Potassium Laurate on B. diphtheriæ.

Salt concentration. (Potassium phosphate.)	Germicidal titers.		
	pH 6.0	pH 7.0	pH 8.0
N/10	N/10,240	N/1,280	N/320
N/25	N/5,120	N/640	N/160
N/100	N/2,560	N/320	N/160

Temperature, 37°C. Time of incubation, 2 hours.

This effect of salt upon the germicidal action of soaps and fatty acids is similar to the effect of salt upon phenol, another organic acid. In the latter case, the effect of salt was explained by Spiro and Bruns (1898) in this manner: When bacteria are added to a solution containing phenol, the equilibrium concentrations of $\frac{\text{phenol in bacteria}}{\text{phenol in water}}$ will depend upon the relative solubility of the phenol in the two phases. If anything is added to the water phase (such as alcohol) that increases the solubility of the phenol in it, then the concentration of phenol in the bacteria is diminished, and likewise germicidal action. Conversely, as salt diminishes the solubility of phenol in water, it increases the phenol concentration in the bacteria, causing increased germicidal action. As salts diminish the solubility of soaps and fatty acids in water, the reasoning of Spiro and Bruns would equally well explain the salt effect here. It should be noted, however, that where the added salt actually precipitates the soap out of solution such enhancement of germicidal action is not to be expected.

If we consider the germicidal titer of soaps for *Streptococcus*, we see that with only one exception (potassium stearate) the different soaps tried were more active in acid than in neutral or alkaline solutions (Figs. 2 and 7). It seems probable that the stearate failed to show this phenomenon because its acid is too insoluble. With *B. diphtheriæ*, *Staphylococcus*, and *V. choleræ*, the palmitate, pentadecylate, and myristate likewise fail to be effective in acid ranges; and with *B. typhosus*, which is the most resistant organism of the group, the tridecylate, laurate, and undecylate are added to the above. In each case it seems likely that the germicidal activity of the fatty acid fails to become manifest because it is not soluble in the necessary concentration. Certainly, in all cases where the acid is sufficiently soluble to show its true germicidal action it is more active than the corresponding soap. In this there is again an analogy with phenol, as Scheurlen and Spiro (1897) showed that phenol is a more powerful germicide than sodium phenolate.

Several factors suggest themselves as being involved in this increased activity in acid solutions:

1. The primary effect of the pH may be on the bacterium rather than on the germicide; an acid reaction may favor germicidal action by rendering the bacterium more sensitive to the soap or fatty acid.
2. An acid reaction may diminish the surface tension of the soap solution, and hence greatly increase the concentration of the germicide at the surface of the bacterium. For soaps containing 12 or fewer carbon atoms this is actually the case. For soaps containing more than 12 carbon atoms the reverse is true (Jarisch, 1922–23). The extreme insolubility of the latter in acid buffer fluids, however, makes surface tension measurements rather unsatisfactory.
3. The effect of the pH may be due to alterations of the solubility of the soaps in the aqueous phase or in the bacterial protoplasm.

This would affect the distribution coefficient of the germicide between germs and test fluid. We do not know what changes occur in the pH of living bacterial protoplasm when the pH of the outside fluid is altered. However, Coulter (1924–25) has shown that when acid is added to a suspension of erythrocytes, the cell contents do not become as acid as the outside fluid. If this is the case with bacteria, an acid outside pH would be accompanied by a much less acid pH of the bacterial protoplasm. As acids diminish the solubility of soaps, an acid reaction of the outside fluid should, therefore, increase the concentration of soap (or fatty acid) in the bacteria. If, however, the added acid precipitates most of the soap out of solution, the actual concentration within the bacteria would be diminished, even though the relative concentration is still greater than that in solution in the outside fluid.

4. The greater germicidal activity of the fatty acid may be due to the fact that the acid is less dissociated than the soap. There is a good deal of evidence to show that undissociated molecules penetrate more readily into protoplasm than do ions (Osterhout, 1925–26). Michaelis and Dernby (1922) believe that the germicidal effect of the organic bases studied by them is due entirely to the undissociated molecules; they show that the curve for the germicidal titer closely parallels the dissociation residue curve for a weak base. Scudder (1914) gives the value of K_a for caprylic acid as 1.44×10^{-5}; the dissociation residue for this acid corresponds very closely with the germicidal titer curves for the same acid (Figs. 1 to 4). The latter, however, are logarithmic curves; hence it would appear that the logarithm of the germicidal titer varies with the dissociation residue of this acid. This means either that other variables enter into the germicidal effect, or that the dissociation residue affects the germicidal titer in two or more different ways (*e.g.* by affecting the surface tension or the distribution coefficient as well as the ability to penetrate).

SUMMARY.

1. The effect of the pH on the germicidal action of soaps has been studied. The lower members of the series were found to be most active in acid solution; the higher members, in alkaline. The point of transition varied with the test organism used.

2. This is probably due to the effect of the pH on the dissociation residue and on the solubility of the soap. The dissociation residue may affect the germicidal titer by modifying the surface tension, the penetration into the bacteria, and the partition coefficient of the germicide between bacteria and water.

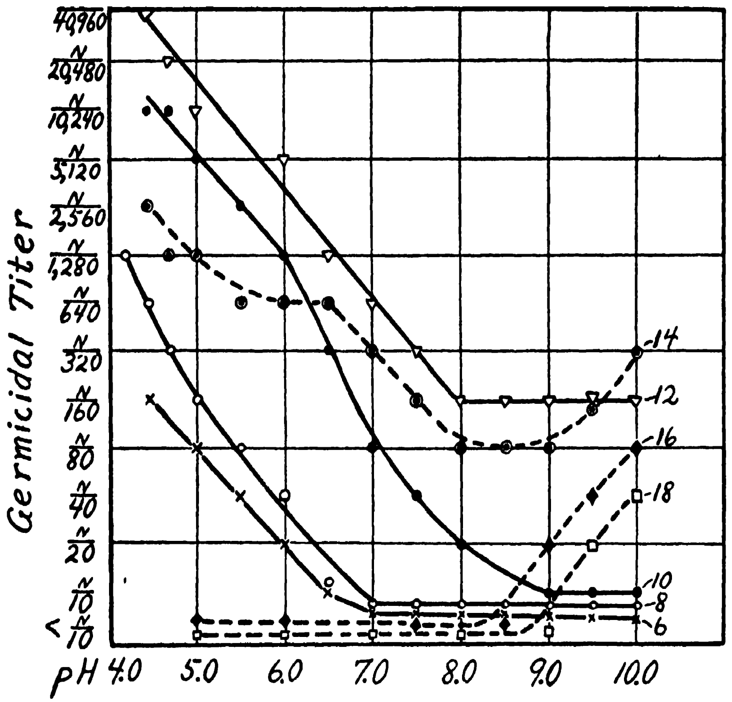

FIG. 1. Germicidal titers of saturated soaps for *Staphylococcus aureus*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate, tridecylate, and pentadecylate are omitted from the figure for simplification; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

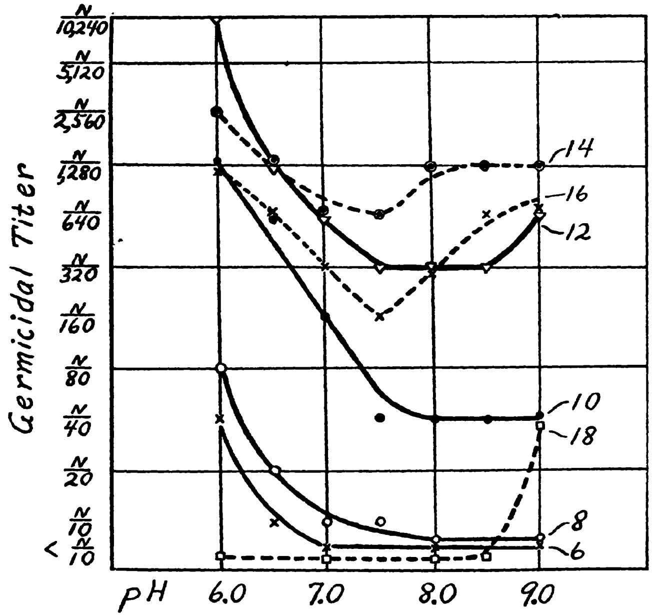

FIG. 2.

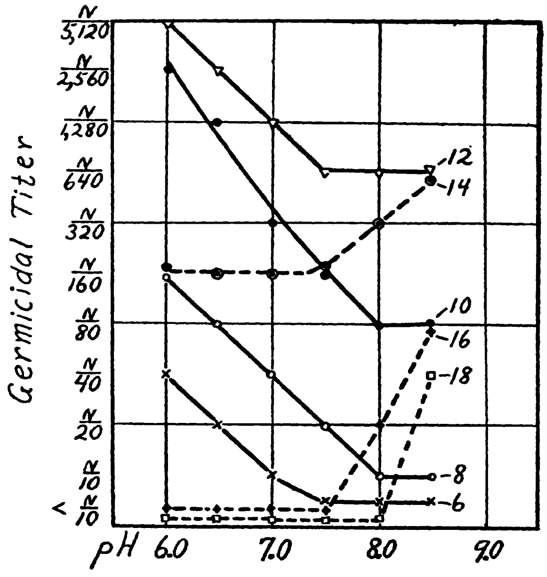

FIG. 3.

FIG. 2. Germicidal titers of saturated soaps for *Streptococcus pyogenes*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate, tridecylate, and pentadecylate are omitted; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

FIG. 3. Germicidal titers of saturated soaps for *V. choleræ*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate, tridecylate, and pentadecylate are omitted; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

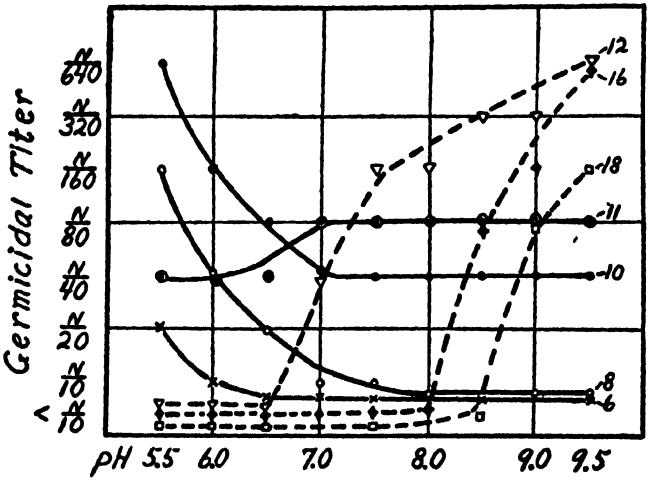

FIG. 4. Germicidal titers of saturated soaps for *B. typhosus*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the soaps with 13, 14, and 15 carbon atoms were omitted to simplify the figure.

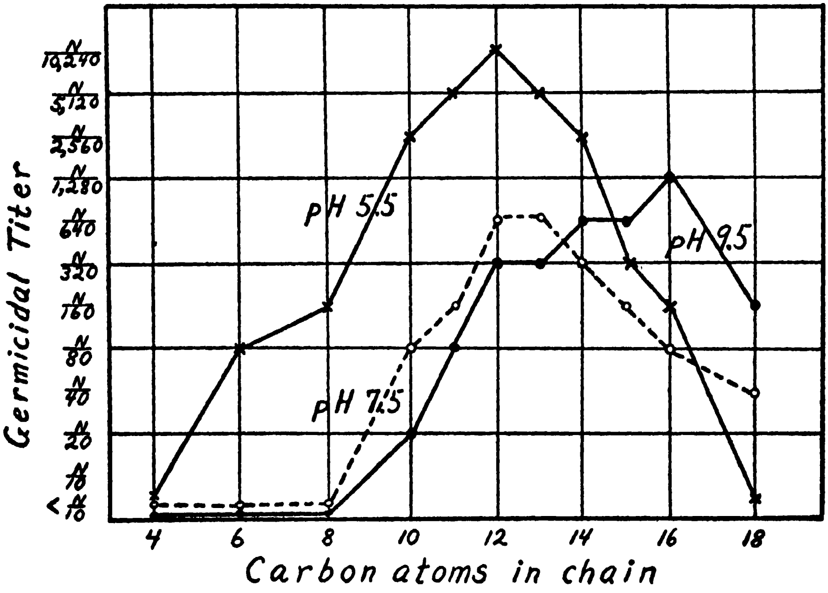

FIG. 5. The germicidal titers of saturated soaps for *B. diphtheriæ*. The incubation period was 2 hours at 37°C. The soaps are designated by the number of carbon atoms in their molecule.

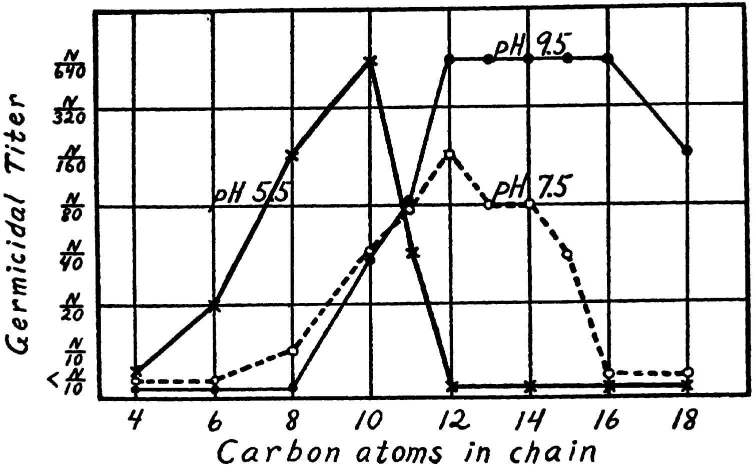

FIG. 6.

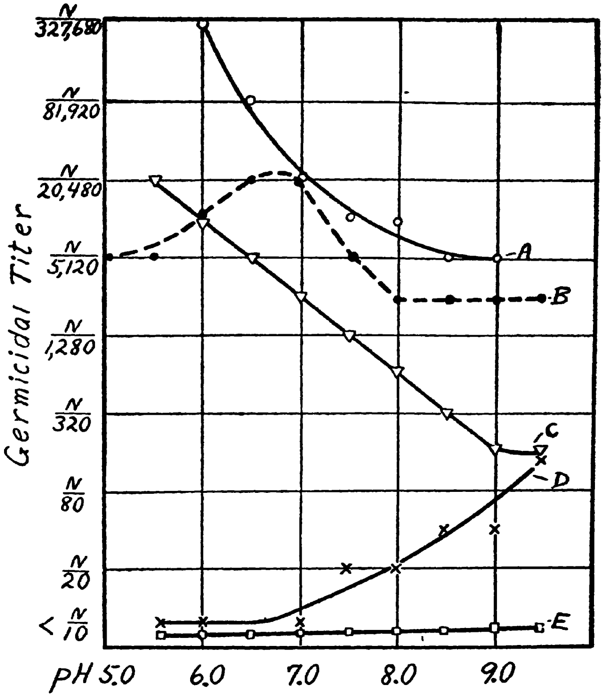

FIG. 7.

FIG. 6. The germicidal titers of saturated soaps for *B. typhosus*. The incubation period was 2 hours at 37°C. The soaps are designated by the number of carbon atoms in their molecule.

FIG. 7. The germicidal titers of two unsaturated soaps. The incubation period was 2 hours at 37°C. *A*. Potassium oleate and *Streptococcus pyogenes*. *B*. Potassium oleate and *B. diphtheriæ*. *C*. Potassium ricinoleate and *B. diphtheriæ*. *D*. Potassium oleate and *B. typhosus*. *E*. Potassium oleate and *Staphylococcus aureus*.

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THE SWELLING PRESSURE OF GELATIN AND THE MECHANISM OF SWELLING IN WATER AND NEUTRAL SALT SOLUTIONS.

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The swelling of gelatin when placed in aqueous solution may be readily separated into three types. The swelling in acid or in alkali and the effect of neutral salts on this swelling have been shown by the work of Procter and Wilson,[1] and Loeb[2] to be due to the osmotic pressure of the ions of the electrolyte, in accordance with the Donnan equilibrium. The initial swelling of dry gelatin in water—which evidently is not connected with the Donnan equilibrium—has been carefully studied by Katz,[3] who was able to show that the heat effects, volume, pressure, and vapor pressure changes were strictly analogous to those observed in the formation of concentrated solutions of many substances, and that the system as a whole behaved as an ideal concentrated solution. The large amount of heat liberated indicates strongly that a reaction occurs between the water and gelatin, resulting, presumably, in the formation of a gelatin hydrate. When sufficient water has been added, however, to reduce the gelatin concentration to less than 50 per cent, the heat effects become very small and yet the gelatin may swell, under favorable conditions, until the concentration of gelatin is 5 per cent or less. It appears improbable that this water is all combined with the gelatin in the form of a hydrate, and it seems necessary to consider this as a third distinct type. It is this type of swelling which is discussed in the present paper.

The influence of salts on this tertiary swelling was found by the writers[4] to be closely parallel to the effect of salts on the osmotic pressure of gelatin solutions, and it was further shown that these effects could not be accounted for on the basis of a Donnan equilibrium. It was suggested that this swelling was a purely osmotic phenomenon and that the effect of salts was due primarily to their influence on the osmotic pressure. It is evident that the problem would be greatly simplified if the pressure with which the water was drawn into the solid gelatin could be measured instead of merely the rate or extent of swelling. This "swelling pressure," although analogous to osmotic pressure of a gelatin solution, cannot be measured in the same way, since if solid gelatin is enclosed in a rigid membrane the pressure will be exerted on the walls of the membrane. It was found possible however, by placing the gelatin outside a porcelain thimble coated with collodion, to measure this swelling pressure. The measurements show that the pressure measured in this way is but little less than the osmotic pressure of the same gelatin when liquid, and they corroborate the idea that the swelling in water and neutral salt solutions is due to the osmotic pressure of the solution held in the meshes of the gel.

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[1] *Cf.* Wilson, J. A., in Bogue, R. H., The theory and application of colloidal behavior, New York and London, 1924, i, 1.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[3] Katz, J. R., *Kolloidchem. Beihefte*, 1917–18, ix, 1.

Experimental Method.

Gelatin.—Isoelectric gelatin was prepared as described by Loeb[2] and all measurements were made at pH 4.7.

The method of making the measurements is shown in Fig. 1. The Chamberland filter was coated with collodion by pouring collodion slowly on the surface while the thimble was rotated mechanically. The tube was partially filled with gelatin of the desired concentration and the thimble and manometer tube, previously filled with water, inserted as shown in the figure. The tube was then placed in a water bath at the desired temperature and the pressure measured after equilibrium was reached. A number of experiments were made to determine whether the thickness of the gelatin layer or the previous treatment of the system had any influence on the final reading. The results of some of these experiments are shown in Table I. The equilibrium pressure is evidently independent of the thickness of the gelatin layer, and the temperature effects are reversible. It was also found that the final pressure was independent of the initial pressure; *i.e.*, it is a true equilibrium value. The results, when the tube was completely filled with gelatin were much more regular, and this method was used in most of the experiments. The figures are the averages of 4 to 8 measurements and are reliable to about 5

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[4] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, viii, 317.

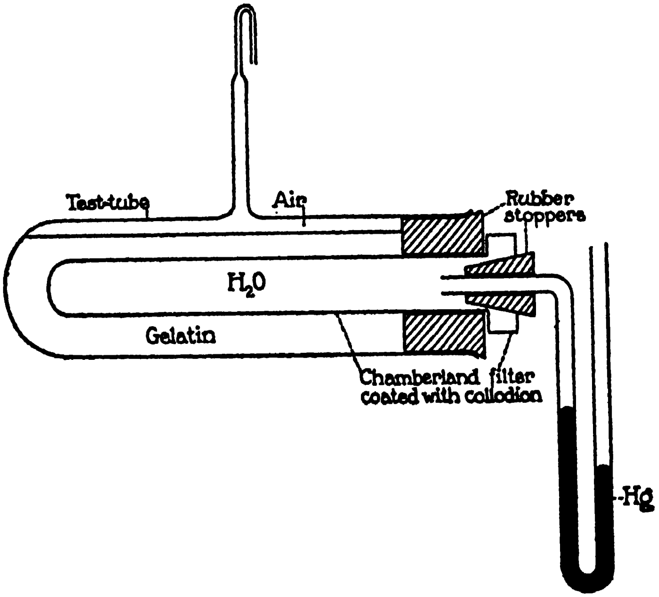

FIG. 1. Apparatus for measuring swelling pressure of gelatin.

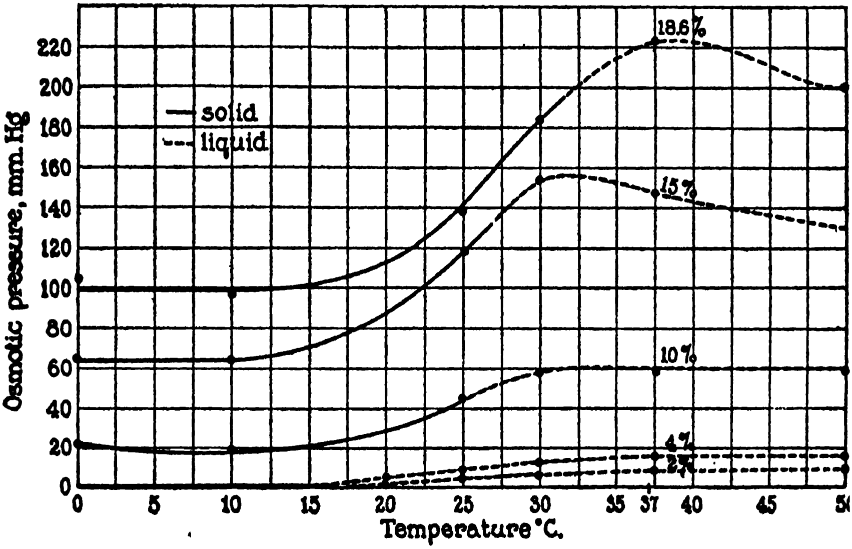

FIG. 2. Osmotic or swelling pressure of various concentrations of gelatin at different temperatures.

per cent. In working with the thin coatings of gelatin it was occasionally noticed that the water evaporated from the gelatin and condensed on the surface of the tube even when the manometer showed high negative pressure on the water in the thimble. This result is exceedingly difficult to account for except on the basis of some temperature difference, and is perhaps similar to von Schroeder's[5] anomalous findings. The pressure of the gelatin when liquid was determined in a rocking osmometer as previously described.[4]

Effect of Temperature and Concentration of Gelatin on the Swelling Pressure of Gelatin.

The result of a series of experiments in which the pressure of various concentrations of gelatin as measured over a range of temperatures is shown in Fig. 2. The solid line indicates that the gelatin was solid and the dotted line indicates liquid gelatin. It will be seen that in high concentrations of gelatin the pressure increases with decreasing temperature to give a maximum near 37°C., and then decreases quite sharply, as the gelatin solidifies, to become nearly independent of the temperature below 15°C. The negative temperature coefficient in the high concentrations is due presumably to the large positive heat of solution[6] and so does not occur in the low concentrations. The drop

TABLE I.

Effect of Thickness of Gelatin Layer and of Previous Treatment on Swelling Pressure of 10 Per Cent Gelatin at 25°C.

Gelatin on thimble.	Other conditions.	Pressure.
cc.		*mm.Hg.*
25	Suspended in closed tube in air.	49
48	" " " " " "	54
195	" " " " " "	44
250	Tube filled with gelatin.	45
250	At 0°C. for 10 min. before placing at 25°C.	48
250	" 0° " " 18 hrs. " " " 25° "	46
250	" 37° " " " " " " " 25° "	47

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[5] von Schroeder, P., *Z. physik. Chem.*, 1903, xlv, 109.

[6] *Cf.* Findlay, A., Osmotic pressure, London and New York, 2nd edition, 1919, 58.

in the pressure between 30° and 20°C. occurs also in the low concentrations where the gelatin does not solidify, and in these cases approaches zero. In these dilute solutions a precipitate forms and hence no osmotic pressure would be expected. If gelatin is considered as a single chemical substance, these results are evidently exceedingly difficult to understand. The drop in the pressure below 35°C. would result if an insoluble substance is separating out, but if the solution contained only this substance the pressure must become independent of the initial concentration as soon as the solid phase appears. This is not the case. The experiment is analogous to Sörensen's[7] results on the solubility of the globulins, in that the osmotic pressure (concentration) increases with the total amount of substance added even in the presence of the solid phase. This is the expected result if it is considered that gelatin is a mixture of (at least) two substances, one of which is easily soluble over the range of temperature studied, and the other of which is readily soluble above 35°C., but much less soluble below 15°C. The drop in the pressure between 35° and 15°C. is due

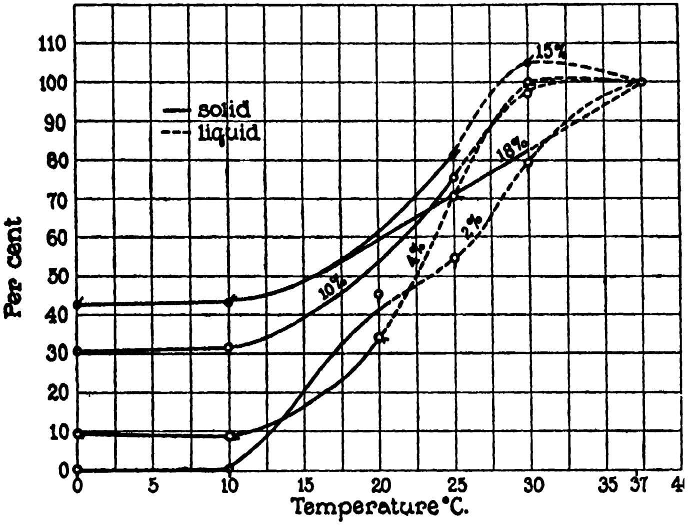

FIG. 3. Osmotic or swelling pressure of gelatin of different concentrations as per cent of value at 37°C.

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[7] Sörensen, S. P. L., Proteins, The Fleischmann Laboratories, New York, 1925.

then to the separation of the insoluble fraction. If the concentration is sufficiently high a gel results; if not, particles are formed containing the soluble material and there is no osmotic pressure of the solution as a whole. The osmotic pressure as per cent of the value at 37°C. is plotted in Fig. 3 and as a function of the concentration of gelatin in Fig. 4. The percentage drop in the osmotic pressure is nearly constant in this range except for the very low concentrations. It will be noticed that the osmotic pressure increases more rapidly than the concentration, a result which can be predicted from the viscosity curves (Kunitz[8]) and which is due to the fact that a considerable quantity of the water is contained in the particles. (This effect will be discussed quantitatively in a subsequent paper.) The pressure also increases with the total concentration even when the gelatin is solid. This shows, as stated above, that the system cannot be considered as a saturated solution of one substance.

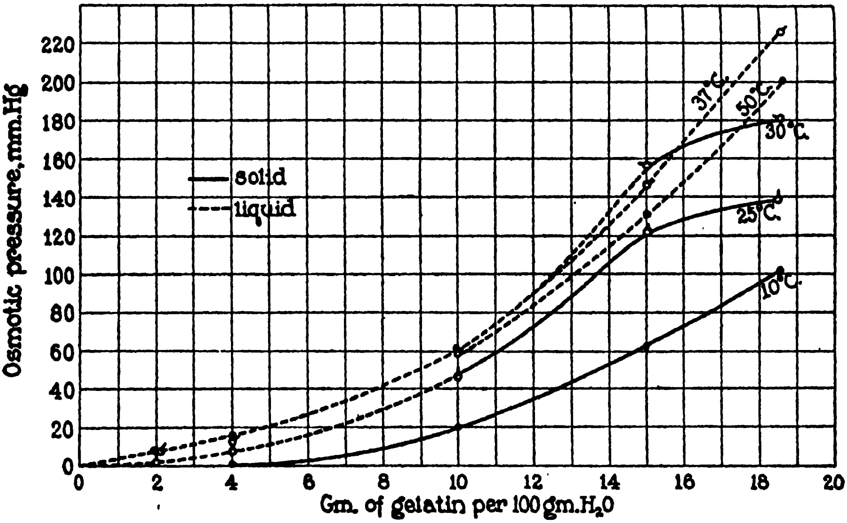

FIG. 4. Concentration and swelling or osmotic pressure of gelatin at different temperatures.

It should be possible, according to these results, to separate gelatin into two fractions: a soluble one, having high osmotic pressure, low viscosity, and no tendency to gel formation, and an insoluble one, having a limited solubility at low temperatures, a low osmotic pressure, and undergoing little or no swelling. It might also be expected, since the soluble fraction must be held in the meshes of the insoluble one, that isolation of the former would be much more easily accomplished than the separation of the insoluble from its accompanying soluble component. It was found possible after a number of attempts to separate gelatin into two fractions having nearly the properties described above. This was done by combining the temperature effect on the solubility with alcohol precipitation. The fractionation was carried out as follows:

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[8] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 715.

Preparation of Insoluble Fraction.

10 liters of 5 per cent isoelectric gelatin at 35°C. and 7.5 liters of 95 per cent alcohol added; 18 hours at 20°C., filtered with folded paper. Filtrate = No. 1.

Precipitate: Made up to 8 liters with water, heated to 35°C., and 2 liters of alcohol added. Cooled to 20°C. and filtered. Filtrate rejected.

Precipitate: Made up to 8 liters, etc. as above, and repeated 4 times.

Precipitate: Made up to 2 liters with water, heated to 35°C., and 1.6 liters of alcohol added. Cooled to 30°C. Jelly-like residue settled. Supernatant liquid decanted and rejected.

Residue: Made up to 2 liters, etc. as above, and repeated 5 times.

Final residue: Made up to 2 liters with water at 35°C., 800 cc. alcohol added, and cooled to 10°C. Precipitate filtered and dried with alcohol and ether. 42 gm. marked "insoluble fraction."

Preparation of Soluble Fraction.

Filtrate No. 1: 24 hours at 0°C. and filtered at 0°C. Filtrate rejected.

Precipitate: Made up to 400 cc. with water, heated to 30°C., 400 cc. of alcohol added, and cooled to 20°C. Filtered. Precipitate rejected.

Filtrate: 18 hours at 0°C. Filtered at 0°C. Filtrate rejected.

Precipitate: Made up to 200 cc., heated to 30°C., and 200 cc. of alcohol added. No precipitate on cooling to 20°C. Cooled to 0°C. 18 hours. Filtered at 0°C. Precipitate dried with alcohol and ether. 25 gm. marked "soluble fraction."

No evidence is at hand to show that either of these fractions is a chemical individual and on the contrary there is reason to suppose that the insoluble fraction still contains a considerable quantity of the soluble since it still swells, and further purification caused a still further

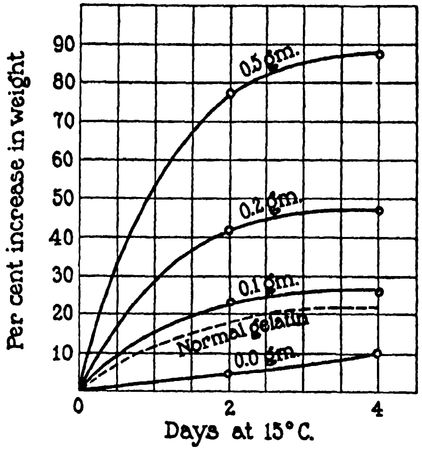

FIG. 5. Effect of increasing amounts of soluble fraction on swelling of insoluble fraction. 5 cc. H_2O, 0.4 gm. insoluble + noted amounts of soluble fraction at 15°C.

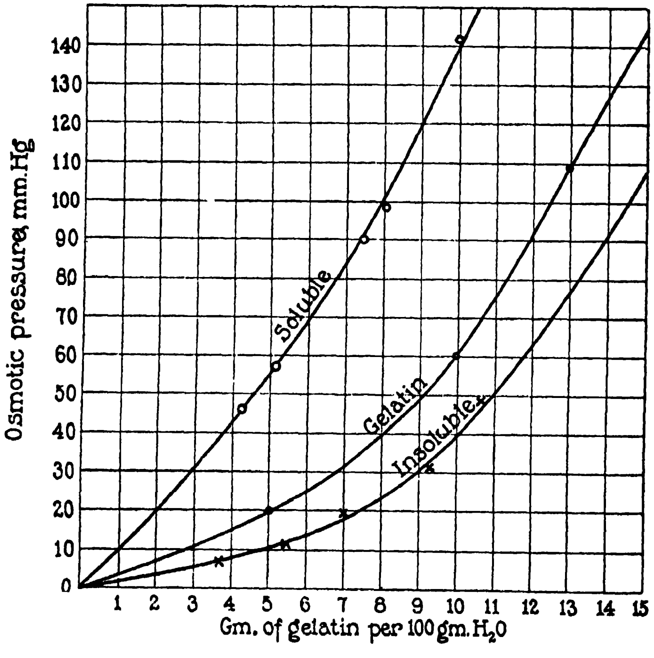

FIG. 6. Osmotic pressure of various concentrations of gelatin and of the soluble and insoluble fractions.

decrease in the osmotic pressure. Owing to the difficulty of obtaining sufficient material with which to work, however, the process was stopped at this stage.

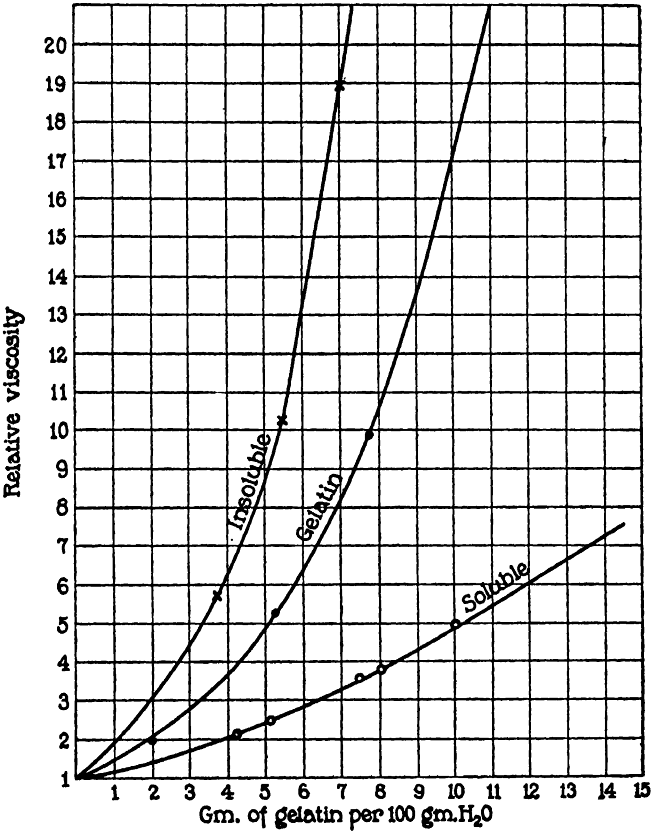

FIG. 7. Viscosity of various concentrations of gelatin and of the soluble and insoluble fractions.

Comparison of the Properties of the Two Fractions and of Gelatin.

In accordance with the mechanism of swelling outlined above it would be expected that the insoluble fraction should swell very little while the soluble fraction should dissolve. Mixtures of the two should swell the more the greater the percentage of soluble material. Fig. 5 shows that this is actually the case. The swelling of the insoluble fraction itself is probably due to the fact that it still contains an appreciable quantity of the soluble material, although it could of course be assumed that a different mechanism was responsible for this swelling.

Figs. 6 and 7 show the results of osmotic pressure and viscosity measurements. The viscosity of the insoluble fraction is much greater than that of gelatin while the osmotic pressure is less. The osmotic pressure of the soluble fraction is greater and the viscosity less, as would be expected. Since the insoluble fraction swells less than gelatin, it might be expected that the viscosity would be less instead of greater. It must be remembered, however, that although the individual particles swell less, the number of particles per gm. is greater in the insoluble fraction than in gelatin, since the latter also contains the soluble material. Since the viscosity, according to Einstein, depends on the total volume occupied by the particles, the higher viscosity of the insoluble fraction is due to the fact that the increase in the number of particles capable of swelling more than makes up for the difference in the swelling of the individual particles. As was stated above, the form of the osmotic pressure curves can be quantitatively accounted for by the absorption of water in the particles. Qualitatively it can

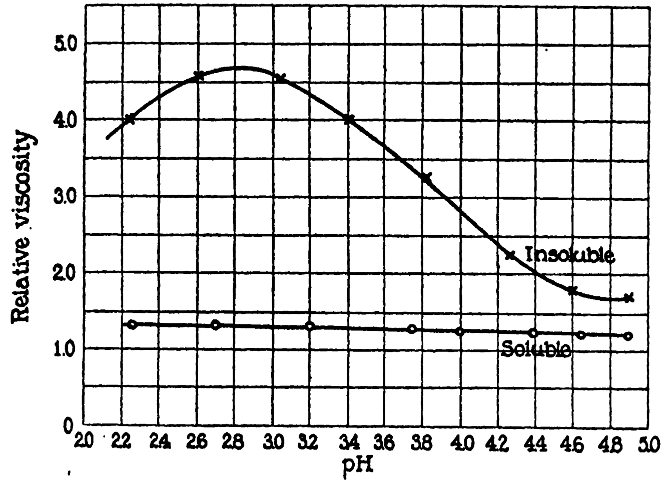

FIG. 8. Effect of addition of HCl on the viscosity of 1 per cent solutions of soluble and insoluble fractions at 37°C.

be predicted that the substance having the highest viscosity should have the greatest curvature in the osmotic pressure-concentration curves and, as the figures show, this is the case. The marked difference in the viscosity of the soluble and insoluble fractions was ascribed above to the fact that the insoluble fraction in solution consisted largely of particles capable of swelling, while the soluble fraction did not. If this is the case, it would be expected that the insoluble fraction should show a marked pH-viscosity effect, while the soluble fraction should show little or none, since, according to Loeb,[2] the increase in viscosity on addition of acid is due to the swelling of such particles by the Donnan equilibrium set up in them. Fig. 8 shows that this is the experimental result. On the other hand, it would be expected that tht effect of acid on the osmotic pressure would be about the same, since in this case the collodion sac is the particle and is evidently present in both cases. This expectation is also fulfilled as is shown in Fig. 9.

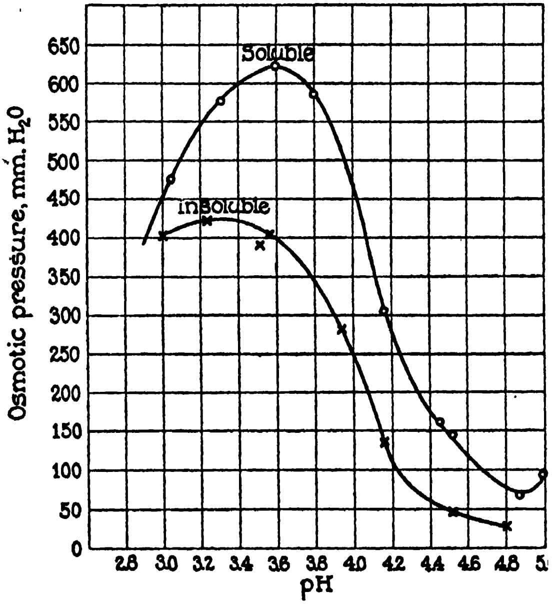

FIG. 9. Effect of addition of HCl on osmotic pressure of 1 per cent solutions of soluble and insoluble fractions at 37°C.

The titration curves of the two fractions, and the speed with which they are digested by pepsin, do not differ significantly from those of ordinary gelatin.

The Effect of Temperature and Salts on the Osmotic Pressure.

It was suggested above that the increase of pressure occurring when gelatin solutions or gels are raised from 25° to 37°C. was due to the solution of the substance forming the network and corresponding to the insoluble fraction. It would be expected therefore that the temperature would have a still greater effect on the osmotic pressure of the insoluble fraction and a smaller effect on the soluble fraction. This is the result, as shown in Table II. The insoluble fraction still exerts pressure below 25°C. which indicates, as stated above, that it still contains some soluble material.

TABLE II.

Effect of Temperature on Osmotic Pressure of Soluble and Insoluble Fractions. 5 Per Cent Solutions.

Temperature.	Pressure in mm. Hg.	
	Soluble fraction.	Insoluble fraction.
°C.		
37	50 liquid.	10 liquid.
25	48 "	6.7 solid.
20		5.3

As is well known, strong solutions of some neutral salts liquefy gelatin and, as was shown in a preceding paper,[4] also increase the osmotic pressure. The action of these salts on gelatin at 15°C., and the effect of raising the temperature to 37°C. are therefore similar except that the increase of osmotic pressure due to the salt is greater than that due to increasing the temperature. A solution of gelatin at 37°C. contains particles capable of swelling, as is shown by the pH-viscosity effect. The osmotic pressure of such a solution could be increased in two ways:

First, the salt might increase the swelling of the particles. Since in this process water is removed, while the number of particles remains the same, the result is an increase in the mol fraction, and a resulting increase in the osmotic pressure. Second, the salt might cause these particles to break up into smaller ones, thereby increasing the number of particles and hence the osmotic pressure. On the basis of the first assumption it would be expected that the salt should also increase the viscosity and further that the effect of temperature, in the presence of salt, would not differ very much from that in the absence of salt. On the second assumption there would be expected no change or a decrease in the viscosity and the temperature effect on the osmotic pressure in the presence of salt should be much less than when no salt is present. The experiments in Table III show that the latter results are obtained. 2 M NaSCN has very little effect on the viscosity of 10 per cent gelatin and the osmotic pressure decreases only slightly in the range from 37° to 3°C. The optical properties of the solution also bear out this as-

TABLE III.

Effect of Salts on Viscosity and Osmotic Pressure of 10 Per Cent Gelatin.

Concentration of salt.	0	2 M NaSCN
Relative viscosity, $\frac{\eta}{\eta_{salt}}$, at 37°C.	19.3	18.10
Osmotic pressure, *mm. Hg*, at 37°C.	60	122
" " " " " 25°C.	44	113
" " " " " 15°C.	20	108
" " " " " 3°C.	20	102

TABLE IV.

Effect of NaSCN on the Osmotic Pressure of 5 Per Cent Soluble and Insoluble Fractions of Gelatin at 37°C.

Fraction.	Soluble.		Insoluble.	
Concentration of NaSCN inside and outside	0	2M	0	2M
Osmotic pressure, *mm. Hg*	50	61	8	35

sumption, since a solution of gelatin at 37°C. shows a very marked Tyndall cone, whereas the same solution in 2 M NaSCN shows only a faint cone.

It has already been shown that the effect of temperature is largely on the insoluble component and it remains to be seen whether the effect of salt is also on this component or on the soluble fraction. Table IV gives the effect of NaSCN on the osmotic pressure of a 5 per cent solution of the soluble and insoluble fractions at 37°C. It is evident that there is a very large increase in the osmotic pressure of the insoluble fraction and only a small increase in that of the soluble one. The salt therefore affects the same constituent as does raising the temperature.

DISCUSSION.

The preceding experiments appear to the writers to furnish a basis for a simple and satisfactory picture of the mechanism of the swelling of isoelectric gelatin in water and in neutral salt solutions. The block of gelatin is a network consisting of threads of a substance insoluble in cold water and holding in its meshes a solution of a substance soluble in water. (For the sake of simplicity only two substances are considered, although in reality there are probably a series of substances whose properties grade from very easily soluble to insoluble.) The process of manufacture of gelatin would be expected to lead to the formation of just such a series of compounds, since it is formed by the hydrolysis of an insoluble substance, collagen. Such a reaction would be expected to produce a series of split products ranging in complexity from the amino-acids up to unchanged collagen. In the further process of purification and washing, all substances which can diffuse through the network of insoluble material are removed while those that cannot are held back. When the block is immersed in water the internal solution exerts osmotic pressure and water is taken in until the osmotic pressure is equalized by the elastic force of the network. A slow subsequent swelling will occur, however, owing to the fatigue of this elastic force. A quantitative expression for the kinetics of swelling has been derived on this basis and will be discussed in a subsequent paper.

Raising the temperature causes an increase in the concentration of the soluble material and hence in the osmotic pressure of the solution and at the same time causes a decrease in the amount of insoluble material forming the fibers. The swelling, therefore, increases very rapidly with the temperature until above 35°C. the network dissolves and the block disintegrates. The insoluble material, however, still exists in the solution in the form of particles capable of swelling, as shown by the pH-viscosity effect, and containing a large amount of water, as shown by the form of the osmotic pressure-concentration curves and the viscosity curves. The addition of neutral salts to the solution acts in the same way as increasing the temperature, except that the material forming the network is broken up into smaller particles.[9] In very high salt concentrations the reverse effect occurs and the gelatin eventually precipitates. The various peculiar hysteresis effects which have been noted in connection with the swelling of gelatin are referable to the effect of the conditions on the elasticity of the network, and are similar to those observed in any elastic body. Cooling the solution results in the reverse process. The particles or molecules of the insoluble material precipitate in the form of a network—if the concentration is high enough—enclosing the solution of the soluble material. If the concentration is low, this network separates in the form of small clots which still contain nearly all of the soluble fraction. It seems quite possible that the formation of this peculiar structure rather than of an ordinary precipitate is due to the presence of the soluble material which acts as a protective colloid, and that a pure preparation of the insoluble material would form a normal precipitate. A very similar condition occurs in the solution and repurification of casein; addition of small amounts of acid or alkali to isoelectric casein results in a normal saturated solution, as Cohn[10] has shown. Precipitation of such a solution by back titration, however, gives a milky colloidal solution which may form a gel, so long as any of the casein remains in solution. When the isoelectric point is reached, where all the casein is insoluble, a normal precipitate is again formed.

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[9] This is perhaps analogous to the prevention of agglutination in strong salt solutions. In the case of bacteria it was found that a marked decrease in the "cohesive force" of the particles was noted in these high salt concentrations. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[10] Cohn, E. J., *J. Gen. Physiol.*, 1921–22, iv, 697.

This mechanism also accounts for the fact that gelatin and similar substances swell only in those solvents in which they are soluble, and it predicts that the greater the solubility the greater the swelling. The swelling which occurs during the early stages of the action of enzymes on gelatin also would be predicted since the hydrolysis increases the concentration of soluble material and hence the osmotic pressure. The swelling caused by enzymes is similar to the effect of salts or higher temperatures except that it is not reversible.

The alternative hypothesis that the effect of temperature or salts is to increase the hydration of the particles and hence the osmotic pressure seems less probable, since it requires that the hydration increase with increasing temperature. Since the hydration of gelatin liberates heat this would be contrary to Le Chatelier's principle. It also fails to predict the increased swelling obtained on adding the soluble fraction to the insoluble, since the osmotic pressure and viscosity curves show that the soluble fraction is less hydrated than the insoluble.

It is evident that the structure of gelatin outlined above is quite similar to that proposed by Hardy[11] and now widely accepted, except that at least two substances are postulated. The whole mechanism agrees in detail with the theory clearly presented by Duclaux[12] in connection with the swelling of rubber.

SUMMARY.

1. A method is described for measuring the swelling pressure of solid gelatin.

2. It was found that this pressure increases rapidly between 15° and 37°C., and that the percentage change is nearly independent of the concentration of gelatin.

3. It is suggested that this pressure is due to the osmotic pressure of a soluble constituent of the gelatin held in the network of insoluble fibers, and that gelatin probably consists of a mixture of at least two substances or groups of substances, one of which is soluble in cold water, does not form a gel, and has a low viscosity and a high osmotic

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[11] Hardy, W. B., *Z. physik. Chem.*, 1900, xxxiii, 327.

[12] Duclaux, J., *Bull. soc. chim.*, 1923, xxxiii–xxxiv, 36.

pressure. The second is insoluble in cold water, forms a gel in very low concentration, and swells much less than ordinary gelatin.

4. Two fractions, having approximately the above properties, were isolated from gelatin by alcohol precipitation at different temperatures.

5. Increasing the temperature and adding neutral salts greatly increase the pressure of the insoluble fraction and have little effect on that of the soluble fraction.

6. Adding increasing amounts of the soluble fraction to the insoluble one results in greater and greater swelling.

7. These results are considered as evidence for the idea that the swelling of gelatin in water or salt solutions is an osmotic phenomenon, and that gelatin consists of a network of an insoluble substance enclosing a solution of a soluble constituent.

THE EFFECT OF pH ON THE PERMEABILITY OF COLLODION MEMBRANES COATED WITH PROTEIN.

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(Accepted for publication, July 28, 1926.)

In a study of some of the factors affecting the formation of protein films on collodion membranes,[1] it was found that the amounts of protein adhering to the membranes varied with the pH in a way similar, in the case of gelatin, to the variation of the fluidity of the protein solutions with the pH. The connection between the viscosity of gelatin solutions and the swelling of gelatin particles had been clearly brought out by Loeb.[2] Dr. Northrop suggested to the writer that it would be of interest to determine whether gelatin and other proteins, when deposited as films on collodion membranes, would exhibit a rise and fall of swelling in acid or alkali, as do granules of gelatin. This has been found to be the case.

EXPERIMENTAL.

The membranes were prepared on mercury by the method already described,[1] about 2 hours being allowed for the evaporation of the solvents through the cardboard case. These membranes were of the most permeable of the types studied in the previous experiments. They were cut into disks 3.81 cm. in diameter, and were coated with protein by being soaked overnight, at 30°C., in the protein solutions. In each case 3 disks were kept overnight at 30°C. in 25 cc. of the solution contained in a wide-mouthed 50 cc. Erlenmeyer flask, and in these experiments the flasks were not agitated. The disks from each flask were washed 3 times with about 300 cc. of water at 30°C. The weights of the disks, with and without protein, were determined after drying overnight at 100°C.

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[1] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–26, viii, 61.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924.

The swelling of the protein on the membranes was studied by measuring the rate of flow, under known pressure, of water and other solutions through the protein-coated membranes in the apparatus previously used,[1] which is similar to that of Bartell and Carpenter.[3] The membrane was bathed on both sides by the solution under investigation, which was forced through the membrane by a constant pressure of mercury. The area of membrane exposed to the liquid was circular, its diameter being 2.2 cm. The rate of flow was measured with the aid of a stop-watch and a mm. scale, by observing the movement of a meniscus in a horizontal tube 0.0760 cm. in diameter. The data were reduced to c.g.s. units and are given in terms of the permeability, Q, which may be defined as the number of cc. of liquid flowing in 1 second through 1 sq. cm. of membrane under a pressure of 1 dyne per sq. cm. The temperature varied from 19 to 24°C. in different experiments.

It was found that solutions of HCl or NaOH up to 0.1 M flowed through membranes which had not been coated with protein at the same rate as water. Hence the differences observed with the protein-coated membranes are to be ascribed to an effect of the electrolyte on the protein rather than on the collodion.

Each experiment was carried out with a single disk of membrane, the determinations being made in the order of increasing concentrations of acid or alkali. Since the different membranes were not all alike, the data of the different experiments are not quantitatively comparable.

Table I shows the effect of different concentrations of HCl on the permeability of a membrane which had been soaked in a 4 per cent solution of isoelectric gelatin. At the end of the experiment 1.0 M NaCl was forced through the membrane, and this was finally displaced with water, which brought the permeability back nearly to the starting point. The experiment shows that while the acid solutions removed very little gelatin from the membrane, they changed the permeability in the direction to be expected if the effect of the acid were on the swelling of gelatin particles.

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[3] Bartell, F. E., and Carpenter, D. C., *J. Phys. Chem.*, 1923, xxvii, 252.

Table II shows a similar experiment with NaOH. Determinations were not made in concentrations above 0.1 M because the alkali attacked the membranes so that they burst under pressure. The changes in permeability are again opposite in direction to the changes in swelling observed by Loeb.

TABLE I.

Effect of HCl on Permeability of Gelatin-Coated Membrane.

Untreated membrane, dry weight = 28.0 mg.; $Q = 26.5 \times 10^{-10}$. At end of experiment, dry weight = 49.5 mg. Control without HCl treatment, 52.0 mg.

HCl, *mols per liter.*	0	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}	3×10^{-1}	1
$Q \times 10^{10}$..	4.22	4.21	4.20	4.18	3.68	2.38	1.62	1.33	1.49	2.63	5.28	7.95

TABLE II.

Effect of NaOH on Permeability of Gelatin-Treated Membrane.

NaOH, *mols per liter*............	0	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}
$Q \times 10^{10}$..........	2.98	2.79	2.03	1.42	1.12	1.06	1.08	1.27

It was found by experiments with HCl and NaOH solutions containing also 0.1 M NaCl that the permeability of similar membranes could be altered by pH even in the presence of salt. Table III shows the results obtained with a gelatin-coated membrane whose permeability was measured in solutions of the glycine-phosphate-acetate buffer described by Northrop and De Kruif.[4] These solutions contained 0.125 M total electrolyte; the pH values were obtained with the hydrogen electrode after the solutions had been used for the permeability measurements. The experiment shows that even in the presence of salt the pH exerts a considerable influence on the permeability, the latter being greatest in the vicinity of the isoelectric point of the protein.

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[4] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

TABLE III.

Effect of Glycine-Phosphate-Acetate Buffer (0.125 M*) on Permeability of Gelatin-Coated Membrane.*

Untreated membrane, dry weight = 27.0 mg.; $Q = 25.7 \times 10^{-10}$. At end of experiment, dry weight = 49.6 mg. Control without buffer treatment, 48.0 mg.

pH..........................	3.45	3.85	4.16	4.95	5.18	5.55	5.97	6.42	6.94	7.41	8.04
$Q \times 10^{10}$.....................	1.63	1.71	2.27	2.47	2.27	2.14	1.86	1.86	1.78	1.74	1.69

Measurements of the permeability of a gelatin-coated membrane in several solutions of HCl and H_2SO_4 showed that the permeability was decreased less by H_2SO_4 than by HCl when the comparison was made at equivalent concentrations. The changes were of the same general nature as those in Table I, but the permeability in H_2SO_4 was always somewhat higher than in HCl. This result is qualitatively in accord with the experiments of Loeb[2] on the swelling of gelatin in acids, although the difference between the effects of the two acids appears to be less in the case of permeability than in the case of swelling. Another membrane was tested in a similar way with NaOH and $Ba(OH)_2$. Again the result was qualitatively similar to the swelling experiments, the permeability being always higher in the presence of a divalent ion of opposite charge to that of the protein.

In order to determine whether the effects observed were confined to gelatin-coated membranes, a few determinations were made of the permeability in HCl of membranes which had been soaked in solutions of egg albumin, edestin, euglobulin from ox serum, and serum albumin from horse serum. A single disk coated with each protein was used for permeability measurements in HCl of the concentrations 0.001, 0.01, and 0.1 M. The amounts of adherent protein on the membranes used were 28 mg. of egg albumin, 4 mg. of edestin, 15 mg. of globulin, and 11 mg. of serum albumin. In every case the permeability was lowest in 0.01 M HCl. The egg albumin and edestin showed only small differences, but in the case of the serum proteins the effect was larger. With serum globulin the permeability in 0.01 M HCl was about two-thirds of that in the 0.001 or 0.1 M

HCl, while with serum albumin and 0.01 M HCl the permeability was less than half of the value obtained with the other two solutions.

SUMMARY.

The permeability of gelatin-coated collodion membranes, as measured by the flow of water or of dilute solutions through the membranes, has been found to vary with the pH of the solutions. The permeability is greatest near the isoelectric point of the protein; with increasing concentration of either acid or alkali it decreases, passes through a minimum, and then increases. These variations with pH are qualitatively in accord with the assumption that they are due to swelling of the gelatin in the pores of the membrane, the effects of pH being similar to those observed by Loeb on the swelling of gelatin granules. Indications have been found of a similar variable permeability in the case of membranes coated with egg albumin, edestin, serum euglobulin, and serum albumin.

TEMPERATURE CHARACTERISTICS FOR SPEED OF MOVEMENT OF THIOBACTERIA.*

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(Accepted for publication, July 8, 1926.)

I.

The gliding movements of certain *Cyanophyceæ*, *Thiobacteriales*, and gregarines provide relatively uncomplicated types of comparable activities suitable for quantitative observation. In spite of much discussion, little is known as to the mechanism of this sort of progression. Interpretations have been advanced by a number of writers,[1] but data required for formulation of a theory of this sort of movement have been lacking. For the present, we are not concerned so much with the mechanics of the movements of *Oscillatoria*, *Beggiatoa*, and other forms creeping in ways apparently similar, but in employing the rate of this type of movement as an index of metabolic changes.

For one kind of *Oscillatoria* it has been shown (Crozier and Federighi, 1924–25) that the rate of translatory movement, in this instance uncomplicated by rotation, obeys the Arrhenius equation for change with temperature. The rates of movement at different temperatures permit evaluation of the constant E, or μ, in the equation

$$\text{Velocity} \propto e^{-\frac{\mu}{RT}},$$

where e is the Napierian base, R the gas constant, and T the absolute temperature. The value of μ obtained in the experiments cited was 9240.

In these observations the light intensity was practically constant.

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* Support from the Milton Research Fund of Harvard University is gratefully acknowledged.

[1] For recent views consult Fechner (1915); Schmid (1918, 1923); Prell (1923, *a* and *b*); Krenner (1925).

To discover the way in which the magnitude of E might be dependent upon light intensity, it was desired to know, among other things, the relations between speed of movement and temperature in a form free from effects due to photosynthetic activity. For this reason, in part, we turned to the leuco-thiobacteria. It is found that the relation of motility to temperature in *Beggiatoa* and *Thiothrix*, as in *Oscillatoria*, points clearly to the controlling influence of chemical processes. The values of the critical increments (E, or μ) in fact agree sufficiently with those repeatedly obtained for other activities of various organisms, and specifically for catalyzed respiratory oxidations (*cf*. Crozier, 1924–25). The value of E derived for speed of movement in *Beggiatoa* thus emphasizes the fact that the magnitude obtained with *Oscillatoria* (Crozier and Federighi, 1924–25) does not accord with any commonly encountered (Crozier, 1925–26, *b*) in connection with biological processes adequately studied. The investigation of the relation between E and light intensity is therefore expected to yield interesting suggestions as to the significance of the critical increment for movement in the case of *Oscillatoria*. This will be discussed in a subsequent paper. In the meantime, it may be pointed out that the movement of *Beggiatoa* appears to be governed by chemical processes similar to those revealed in a number of other vital activities; the details of the relationship between speed of translatory movement and temperature show certain features of general interest for this method of analysis.

II.

The organisms employed for the measurements were kept in shallow culture dishes containing the usual variety of forms occurring in brackish water putrefactive sulfureta (*cf*. Bavendamm, 1924; Baas-Becking, 1925). Two species, identified as corresponding to *Beggiatoa alba* and to a species of *Thiothrix* (*T. tenuis ?*), were taken for study from particular spots in one culture. Thin smears were mounted between two cover glasses, the lower one small enough to be placed within the glass ring of a van Tieghem cell. The cell was sealed with paraffin or chicle, and had in it a small volume of the culture liquid. The sealing was necessary to prevent dilution when the preparation was submerged in a thermostat; access of tap water caused cessation of progression movements. The cell was mounted in a mechanical stage on the platform of a microscope so adjusted as to have the preparation submerged to a depth of 10 cm. in a water thermostat. The mechanical stage and the fine adjustment of the microscope were controlled by suitable attachments projecting above the water level. With good stirring no difficulty was experienced in maintaining desired temperatures. Light from a housed tungsten bulb was reflected from a mirror beneath the microscope. Variations in light intensity were apparently without effect on the movements of the sulfur bacteria, but for practically all of the measurements the light was of approximately 30 m. c. intensity. Within periods of 6 hours or longer, even up to 24 hours, no progressive changes in speed of movement were detected. Hence the sealed atmosphere in the observation cell produced no special effect.

The measurements were made of the longitudinal progression of straight filaments, and so far as could be determined in the absence of mechanical impedance. With a 5 mm. objective and 7.5 × ocular, ten divisions of the ocular micrometer used corresponded to 0.05 mm. The time required for each filament to traverse this distance was taken with a stop-watch, a number of readings being secured at each temperature. With each preparation used, precautions were taken, through time records and by reversing the sequence of temperature changes, to insure the absence of irreversible thermal effects.

III.

According to the current understanding of the mechanism of movement in *Oscillatoria* the longitudinal membrane (Hinze, 1902) of a filament is pierced by pores, through which a carbohydrate mucus is extruded (Fechner, 1915; Schmid, 1918, 1923; Prell, 1921, *a;* Krenner, 1925; Ruhland and Hoffmann, 1925). This would account for the phenomena which gave rise to the older conception of "extracellular protoplasmic streaming." Another view regards the movement as due to "modifications of surface tension," perhaps caused by osmotic processes (Coupin, 1923); though suggestive, no particularly relevant evidence supports this idea. Krenner (1925) found the speed of translatory movement of *Oscillatoria* to vary inversely with the diameter of the species, and that the osmotic pressure of the protoplasts is higher in the narrow forms (measured by plasmolytic shrinkage method). Krenner therefore supposes that the specific speed of motion is determined by the turgor. For *Oscillatoria* and its relatives it is known that in general the stouter forms are the more slowly moving. On this basis, one might rather expect the specific speed to be determined by some relationship of surface to bulk. But among the sulfur bacteria we find that with forms occurring side by side in the same culture, the larger species move more quickly,—for two forms, in about the ratio of 1 to 1.5, at the same temperature, when the filament diameters are in the ratio 3.22:1.

We are by no means clear as to the meaning of the optical evidence for "extracellular protoplasmic streaming" (*cf.* also Crozier and Federighi, 1924–25; and Krenner, 1925), nor as to the homology of the superficial slime-covering in *Beggiatoa mirabilis* (Hinze, 1902; Ruhland and Hoffmann, 1925), which we have also observed, with surface structures in the forms we have employed for measurements of speed of movement.

According to Schmid (1923), who studied fragmented filaments, all parts of a filament of *Oscillatoria* are motile. Prell (1921*a*, *b*,) found that the cells of a filament "cooperate," although there seems to be no conduction of stimuli from one part of a filament to another. This agrees with the observation (Crozier and Federighi, 1924–25) that the speed of movement does not vary with the length of the filament. Mr. E. S. Castle has made similar observations on *Anabæna.* It has been noticed, however, that very small groups of cells do not move (Krenner, 1925). In *Beggiatoa* very short fragments, even comprising but three to five cells, do move, but only for very short distances; the the frequency of reversal in direction is very high. It is to be noted, as bearing upon unity of action in long filaments, that there is frequently apparent a failure of the parts of a filament to cooperate. With very long filaments (2 mm.), the two terminal regions may be moving in opposite directions; or a hook bend at one end may be moved forward bodily, in such fashion as to indicate that the bent tip region is not at all contributing to the movement. Similar cases occur in which reversal of direction of movement is not synchronous over the whole filament (*cf.* also Keil, 1912). Aside from their bearing upon the mechanism of movement, these points are of practical moment for the measurement of speeds of progression under comparable conditions.

The speed of movement declines as the culture containing the thiobacteria ages and the cells of the organisms become vacuolated. During the most active period of growth the speed of translatory movement is quite sufficiently uniform to permit significant measurements. The speed is independent of the length of the filament. Successive estimations with a single filament show satisfactory constancy, as may be illustrated by several sets of readings:

Filament.	Temperature. *t*	Time to travel 10 micrometer divisions.
	°*C.*	*sec.*
A	10.2	34.6
		38.6
		39.8
		39.0
		36.8
B	16.8	23.8
		22.2
		21.8
		22.2
C	19.3	14.4
		17.6
		17.2
		18.0

It could not be shown that these slight variations are related to the incidence of reversals of direction.

The latitude of variation in such series does not significantly differ from that in series obtained from a number of different filaments. This was tested sufficiently to ensure the possibility of employing averages based upon measurements with a number of filaments. It is practically impossible, however desirable, to obtain readings over a range of temperatures from single filaments. There is indication of fluctuating variation in speed of movement, not correlated with time of day, in which the period is rather long. This is in part responsible for the scatter of the plotted means (Fig. 1). The probable error of the plotted means is less than 5 per cent of the corresponding means (usually less than 4 per cent). For purposes of the present account we have employed data from filaments in one culture, between January 14 and January 28, 1926. Throughout this period no systematic changes in speed of movement were detected. The number of observations was 431.

IV.

The results are plotted in Fig. 1. Contrary to the case of *Oscillatoria* (Crozier and Federighi, 1924–25), the motion of *Beggiatoa* exhibits a sharp change in the relation to temperature at about 16.5°. The lines providing a satisfactory fit to the two portions of the log speed–$1/T°$ *abs.* graph have slopes respectively, yielding $\mu = 8{,}400$ and $\mu = 16{,}100$ as temperature characteristics.

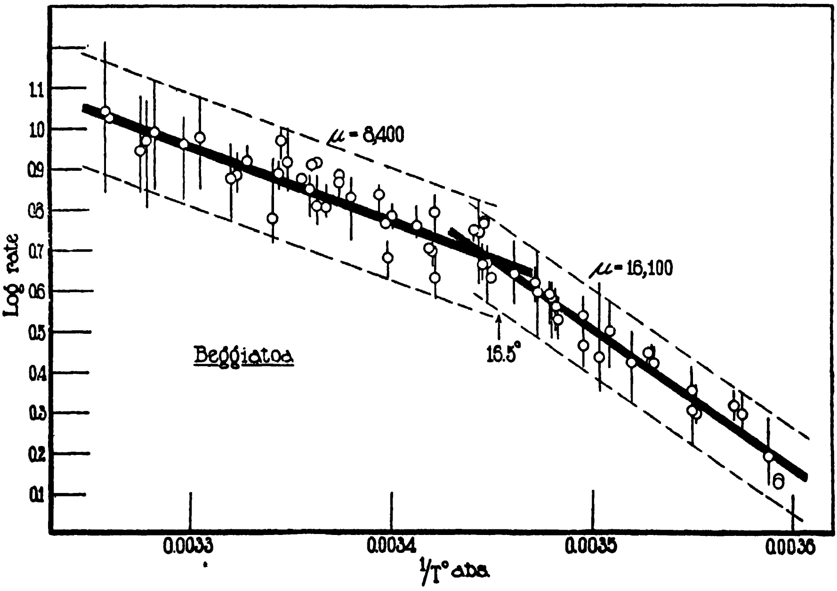

Fig. 1. Speed of gliding motion of *Beggiatoa* as function of temperature. The circles give positions of average speeds in series of readings; the vertical lines extending from them cover the latitude of variation in each series. The rate is taken as 100/ time to travel 10 micrometer divisions (0.05 mm.).

It may be suggested that the data are equally well fitted by a single unbroken curve. To this there is definitely opposed the fact that in such a case the slopes of the fitted straight lines could not very well be expected to agree with those found in numerous cases where a single rectilinear relationship holds over the whole of the temperature scale. The impossibility of describing such series of observations by means of a single smooth curve is adequately shown by plotting *rates* against centigrade temperatures; the points fall upon two sharply intersecting curves. And there is also to be emphasized the fact that the temperature at which intersection of the proposed straight lines is located, as determined solely by the distributions of the relevant points, agrees so closely with one at which such irregularity is commonly or very frequently manifest in other vital processes (Crozier, 1925–26, *b*). Additional considerations justifying this procedure are discussed in another place (Crozier and Stier, 1926–27). Less extensive observations on the movement of *Thiothrix* provide data for the graph in Fig. 2. The temperature characteristic, μ = 8,300, agrees well with that for the corresponding temperature range with *Beggiatoa*.[2]

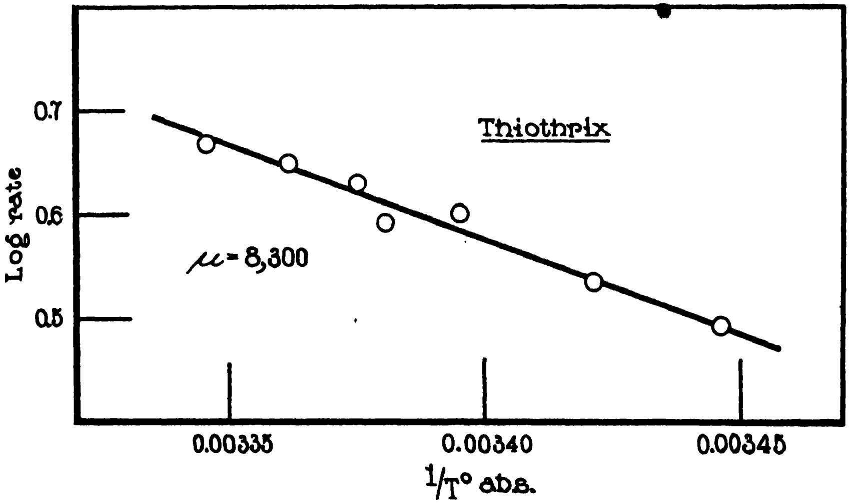

FIG. 2. Speed of movement in *Thiothrix* as related to temperature. The points are averages of 6 to 8 measurements each.

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[2] As with *Oscillatoria*, question also arises here as to the character and mechanism of reversal in direction of movement. According to Coupin (1923) *Oscillatoria* filaments, on Knop medium to which gelose had been added, show no regular periodicity in the reversal of movement; but it is necessary to maintain constant conditions of light and temperature before the matter can be tested. It is clear

In addition to the occurrence of a critical "break" at 16°, the temperatures 5.3° and 33° were established at points at which progressive slowing of movement with time becomes evident; at 33° or above "jerky" side to side movement is evident, with little forward motion.

The "break" at 16° is made obvious in another way. The latitude of variation at temperatures below 16° is definitely less than at higher temperatures. For some time it has been desired to find instances in which it might be possible to discover if the latitude of variation is a property of the organism or tissue as a whole, or of the process whose critical increment is being measured. It is clear, we believe, that in general, and depending on the nature of the activity considered, both these types of variation must be recognized as possible. In many instances it has appeared that the latitude of variation may change without affecting the temperature characteristic (*e.g.*, Crozier and Stier, 1925–26, 1926–27); on the other hand, the latitude may be sensibly constant when the increment changes. The present case is one in which there is apparent alteration of the latitude accompanying a change of increment.[3]

V.

SUMMARY.

The speed of translatory movement of *Beggiatoa alba* is governed by temperature in such a way that between 5° and 33° the temperature characteristics $\mu = 16{,}100$ and $\mu = 8{,}400$ respectively obtain for the temperature ranges 5° to 16.5° and 16.5° to 33°. The "break" at 16°–17° is emphasized by the occurrence of a wider latitude of variation in speed above this temperature. Above 16° the progression of *Thiothrix* yields $\mu = 8{,}300$. The possible relation of these values to that previously obtained for similar movement in (photosynthetic) *Oscillatoria* is commented upon.

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that the frequency of reversal is related to the length of the filament, being greater with short filaments, and certainly it increases with elevation of temperature. Reversal is more frequent in *B. alba* than in *Thiothrix*, under the same conditions. In forms we have observed the frequency of reversal has a higher temperature coefficient than the speed of translation.

[3] This may also be the case with the locomotion of *Paramecium* (Glaser, 1925–26).

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STEREOTROPISM IN RATS AND MICE.

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I.

With diplopods (Crozier and Moore, 1922–23) and with larvæ of *Tenebrio* (Crozier, 1923–24, *a*, *b*) it has been shown that posterior unilateral contact of the creeping animal with the edge of a thick glass plate forces the head to turn in the direction of contact. Contact with two lateral surfaces of equal extent prevents stereotropic bending, and the animal proceeds in a straight path. It was also found, especially with *Tenebrio* larvæ, that stereotropic orientation due to unilateral contact, particularly at the anterior end, persists briefly after cessation of the contact; and that unequal bilateral contacts lead to orientation through an angle roughly proportional to the difference in areas of contact.

These observations can be repeated with a variety of forms, land isopods among others, and show the purely tropistic character of such orientations during creeping. The present experiments are concerned with rats and mice. In all essential details the results duplicate those obtained with arthropods. Their special interest lies in the fact that they demonstrate the occurrence of simple tropistic behavior in mammals. The effects of memory images (Loeb, 1918), and of the great enrichment of sensory fields and their central projections (Parker, 1922), enormously increase the number of possible responses which higher vertebrates may exhibit, and prediction of the course of movements is correspondingly restricted. In order to observe tropistic conduct in a mammal, it is necessary to deal with a type of response which dominates the animal's conduct so strongly as to exclude the influence of stimulations not directly connected with this particular aspect of behavior. The stereotropism of young rats and mice fulfills this condition.

The behavior of the young opossum at birth appears to give an instance of geotropism. After being licked free of blood and embryonic membranes, they climb "hand over hand" from the genital opening to the pouch (a distance of about three inches), and attach themselves to the teats (Hartman, 1920). "If the skin be tilted, the embryos can be made to travel upward and even *away* from the pouch for they are negatively geotropic." The postural reflexes of the decerebrated rabbit (Magnus, 1915–16) include responses which may be taken to have a basis in stereotropism. If placed on the ground with the body in an asymmetric position, the head moves to the normal symmetrical orientation even in the absence of otic labyrinths. This is prevented if a board is placed upon the animal lying in an asymmetrical position; when asymmetric contact-stimulation is equalized, the animal retains an abnormal position.

Accounts of the behavior of rats and mice (*e.g.* Vincent, 1911–12) contain a number of observations suggesting stereotropic guidance.[1] We desired to see if tropistic conduct could not be demonstrated more clearly. This is best accomplished by determining if, as in the case of invertebrates, stereotropic orientation during creeping might not obey the law of the composition of forces. In this event equal bilateral contacts should obliterate turning toward a source of contact. To rule out effects of vision and of the tactile rôle of vibrissæ, we have used animals lacking eyes or vibrissæ. It is neater, in so doing, to avoid experimental mutilation by employing individuals "operated upon" through the agency of a genetic factor, such as results in blindness. This we have been able to do.

II.

The animals used were young rats (*Rattus norvegicus*) and mice (*Mus musculus*) aged 9 to 20 days. The rats were albinos and dark-eyed young from a backcross of the King inbred albinos to hooded rats sired by King inbred males. The mice were all dark-eyed.

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[1] Watson (1914, p. 424) seems to express a view generally held, that "the so-called 'stereotropism' which such animals exhibit is probably no more a case of stereotropism than is the action of a blind man in keeping near a wall or the edge of the side walk." On the preceding page, moreover, he considers it "strange" that when vibrissæ are removed from the *right* side, the rat keeps close to the *left* side of the path in a maze. *Cf.* also Przibram (1913, p. 99).

The eyes of young mice and rats are opened 10 to 14 days after birth. At first, only animals with unopened eyes were used; but it was soon found that young animals with opened eyes gave substantially the same results. Tests with rats were for the most part made in a dark room, under red light of low intensity, at 23–25°C. Experiments with mice were made at about the same temperature, but not in a dark room. Temperatures as low as 15° greatly reduce activity.

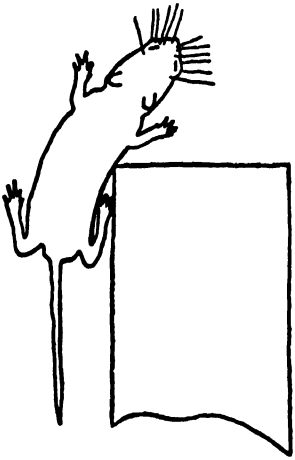

FIG. 1.

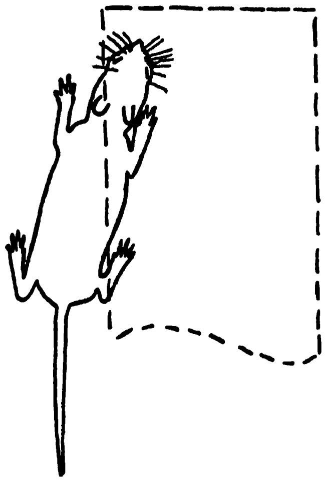

FIG 2

FIG. 1. Stereotropic orientation of young rat or mouse at the corner of a box along one side of which it has been creeping.

FIG 2. A young rat or mouse has been creeping in contact with the side of a box (dashed outline); the removal of the box results in partial orientation toward that side.

Typical stereotropic behavior is observed in animals creeping or walking at a fairly rapid rate. During slow progression there is more opportunity for the lurching gait to induce movements which, while in the main of stereotropic origin, nevertheless interfere with diagrammatic orientation.

Contact with a vertical surface during creeping results in its being followed closely, and at the end of the surface bending is invariably seen toward the contact side. Depending upon the rate of creeping, the animal either proceeds at an angle with the path while in contact, or if the progression has been slow, it may turn and continue to maintain contact. Fig. 1 shows the path taken after contact with the side of a box, and at its corner. If the box is suddenly removed while the animal is creeping, there is always a swerving toward the side where the box was located (Fig. 2). Stroking one side causes turning in that direction. These results are exactly similar to those gotten with arthropods.

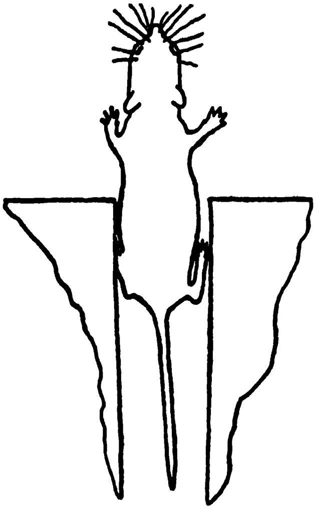

FIG. 3. A young rat or mouse creeping in a passage-way between two boxes, just wide enough to permit gentle contact on either side during the animal's swaying progression, is found to emerge from the passage-way without orientation. Equivalent bilateral stimulations prevent stereotropic turning.

When the vibrissæ have been recently cut away the creeping movements are slower and more uncertain, yet the stereotropic responses continue; several days later the uncertainty of the creeping is lost, but the animal continues to move with head held close to the floor. Removal of the tail has even less effect. Removal of both tail and vibrissæ does not materially interfere. The surface of the body and legs is thus sufficient to control stereotropism.

III.

A young rat or mouse creeping between two boxes so placed as to give equal contact on either side typically emerges from the alley-way in a perfectly straight course (Fig. 3). The bulging of the body at the level of the hind legs, coupled with the lurching gait, sometimes causes quite unequal contacts on the two sides, and this results in modification of the path on emergence. If one box is advanced beyond the other, the animal frequently emerges at an angle toward the extended side. This angle decreases with increase of the excess contact zone on that side. If one surface extends more than the length of the body beyond the corner of the opposed box, the mouse or rat emerges at a very acute angle and then orients so as to round the corner against the surface of the more extended box (Fig. 4). This is not exactly the

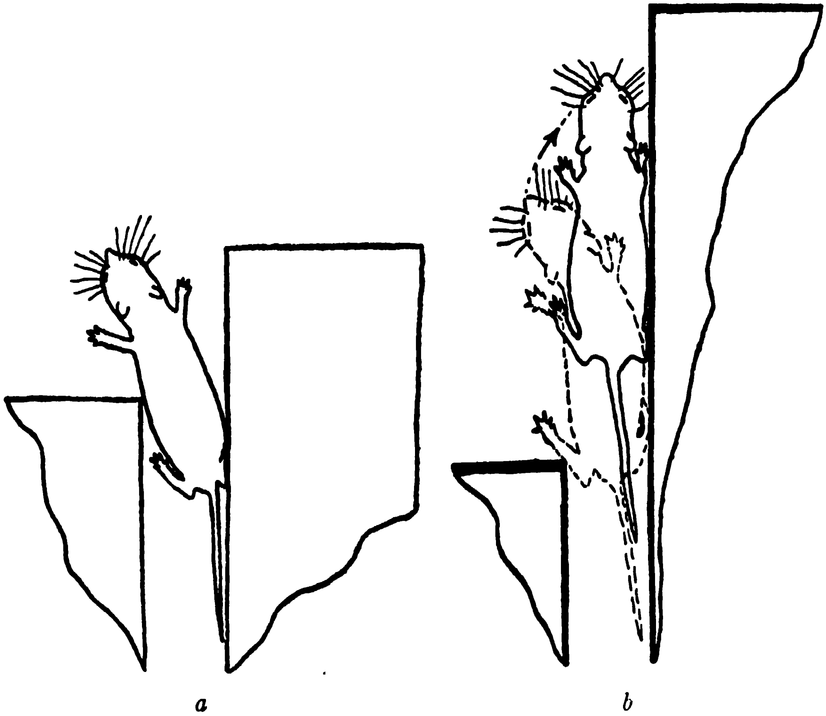

FIG. 4. *a*. Contact at one side with the corner of a box may lead to orientation toward that side, apparently due, in part at least, to more intense tactile excitation than is provided by a continuous flat surface (or by smoothly rounded corners; see Fig. 5).

b. When such a corner is passed, orientation persists toward a continuing contact on the opposite side.

result obtained with arthropods and other forms, which emerge at an angle toward the side of more extensive contact. But further tests show that this at-first-sight anomalous result is due to the relatively excessive tactile stimulation provided by the sharp corners of the wooden boxes used in such experiments. The same outcome is generally observed if thick blocks of paraffin are used instead, provided they have sharp corners. But if the corners be smoothly rounded, the result of such tests is entirely consonant with the interpretation that stereotropic orientation during creeping varies in amplitude according to the difference in the excitations on the two sides (Fig. 5). If one of two opposed boxes providing bilateral contact at emergence be suddenly removed, the animal orients toward the remaining surface (Fig. 6).

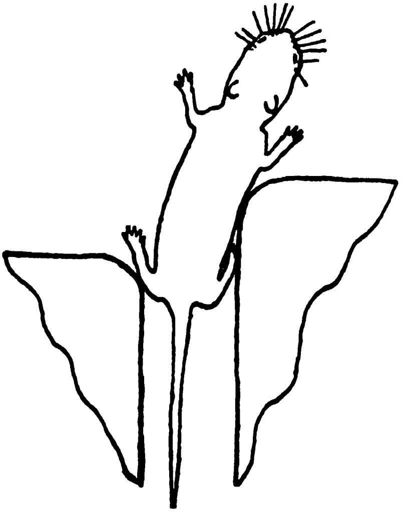

FIG. 5.

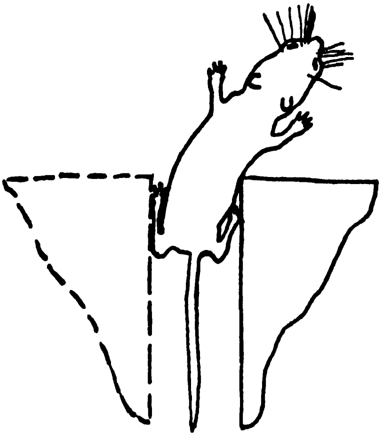

FIG. 6.

FIG. 5. When blocks providing lateral contacts are of unequal extent, the young rat or mouse orients toward the side of more extensive contacts, but does not completely turn the corner unless the difference in extent of the two blocks is more than half the length of the animal. This, the expected result from a tropistic standpoint, is obtained when the corners of the contact blocks are smoothly rounded.

FIG. 6. An individual emerging from equal bilateral contacts with two boxes (*cf.* Fig. 3) proceeds in a straight path, without orientation; but if one of the boxes be removed (dashed outline), it promptly orients toward the remaining one.

These experiments were repeated many times, with particular effort to obviate any persistent tendency of single individuals to right- or left-hand turning.

During creeping in contact with a single vertical surface, the young rat or mouse, especially if moving very slowly, occasionally reverses direction. Observation shows that this occurs when the opposite side makes contact with the floor, as the animal falls into the "corner" between the floor and box; this is similar to the rotation of the body on its long axis observed with invertebrates creeping in the angle between a vertical and a horizontal surface. As a rule, the area of contact is then greater on the side toward the box, and orientation in this direction results in reversal of the path.

IV.

These responses have also been obtained with adult mice and rats, but visual and other sources of stimulation frequently make them much less precise.

Mice blind through hereditary defect characterized by absence of visual cells in the retina were very kindly loaned to us by Dr. Clyde Keeler of the Bussey Institution (*cf.* Keeler, 1926). The locomotion of these adult mice is much more direct than in the case of the very young individuals, and the typical stereotropic responses were obtainable with great certainty and clearness.

V.

The reactions we have described as typical are of course not exhibited with diagrammatic clearness at every trial. The more significant sorts of deviation, however, are themselves stereotropic in origin.

The stereotropism of rats and mice as observed in these experiments was always positive. Movement away from a contact surface is occasionally seen with the younger animals, but it is easily shown that this is an accidental consequence of the method of creeping. The leg muscles are not yet well developed, and the legs are disproportionately long; the body is kept fairly close to the ground and the legs are advanced in a way which cause the rather unsteady creeping act to be a succession of pronounced lurches. An occasional lunge removes the animal from a vertical contact surface, and if all contact has been lost it may creep away from it; usually, however, the residual effect of the contact surface is sufficient to cause reorientation toward it. In case complete separation from the vertical surface has not been followed by reorientation and return, test by contact with a new surface always shows that the animal is still positively stereotropic.

The swaying mode of progression may cause a young rat emerging from bilateral contacts to move toward one side. Thus in one series of trials, with five rats aged 20 days, each animal passed eight times between two vertical contact surfaces of equal extent; in another series eleven rats aged 12 to 14 days each passed five times through equal vertical contact zones; in thirty-one of the first forty tests, and in forty-four of the second lot of fifty-five tests, emergence was in a straight line; in the cases of deviation toward one side, it was seen that the divergence was due to a lunging in that direction rather than to an act of orientation.

When the animal moves at a fairly rapid rate the unilateral effects of lurching movements are more or less equalized. With older individuals the stronger legs make for a straighter course and the influence of lurching motion almost completely disappears. Thus animals about 25 days old show extremely regular reactions, as do the adult blind mice.

The chief sources of apparent irregularity in the stereotropic responses is found to lie in chance contacts of tail and especially of vibrissæ with the boxes employed to give contact surfaces. These variations are reduced by removal of vibrissæ and tail, but the slowness and uncertainty of progression subsequent to these operations introduce other complications and prevent precise measurement of the relation of unequal contact on the two sides to the angle of orientation.

VI.

SUMMARY.

Typical stereotropic orientation toward a lateral surface of contact is obtained in young rats and mice, and with adult mice congenitally blind. Removal of vibrissæ or tail or both does not essentially affect this response.

Equal contact on both sides of the body prevents orientation toward either source of contact. Unequal contact areas on the two sides leads to orientation toward the more extensive contact.

This behavior very exactly parallels the stereotropic conduct of arthropods, and thus provides a fairly complete instance of a tropism in mammals.

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HUMAN GROWTH CURVE.

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1. Statement of Problem.

The fascinating changes in the velocity of development, as well as in proportion of parts, that the growing child shows have long been watched with interest by parents but have only lately been made the subject of scientific analysis. Quetelet (1870) was perhaps the first to measure children at each year of age; but his subjects were few in number (ten to each year), strictly, but not always wisely, selected. Then came the measurements of great numbers of school children by Bowditch (1875) in Boston. This work was followed by a host of similar investigations whose results are summarized by Baldwin (1921) and in my 1926 paper.

The first attempt to interpret the course of human development on a chemical basis was made in the same year by W. Ostwald and by T. Brailsford Robertson, the latter of whom has published a remarkable series of papers since 1908. Robertson early concluded that there are three maxima in the curve of growth of man; one intrauterine, a second that reaches its greatest velocity at about the 6th year, and a third which, in the male, occurs at about the 16th year. This view of a triple set of growth cycles is still adhered to by Robertson who discusses them fully in his book Chemical basis of growth and senescence (1923) and later papers. Robertson's conclusions have been largely based on the data published by Quetelet, and this selection has not been altogether fortunate. Brody has extended Robertson's methods of analyzing the growth curve; but recently (1926) he has found the human growth curve to be of a different order from the growth curve of other mammals and he has been led to abandon, for the present, attempts at its interpretation. In view of the unsatisfactory condition of the analysis of the human growth curve it has seemed desirable to reattempt it, using the best available data. This is the excuse for the present paper.

2. *Methods and Material.*

The curve of development of weight from conception to maturity (Fig. 1) is based on data drawn from various sources. For the antenatal portion the data of Streeter (1920) have been utilized. For postnatal weights, up to 6 years, the data of Woodbury (1921) have been used. For later years various sources, chiefly Nordic males as given in Table B of my Human metamorphosis (1926), were used.

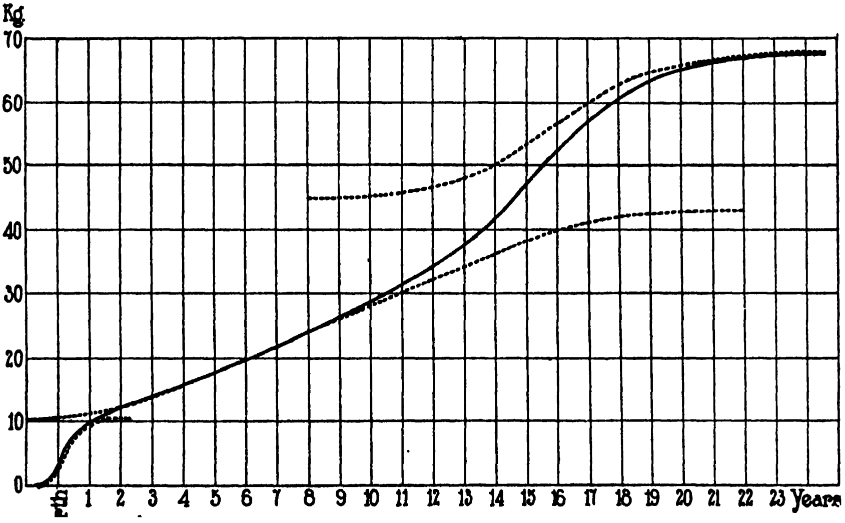

FIG. 1. Analysis of the developmental curve of body weight (full line) into two auto-catalytic curves (dotted line at top and bottom) and a residual curve (dotted line in the middle). Human Nordic stock, males. The autocatalytic and residual curves drawn in free-hand. Abscissæ, time in years; ordinates, body weight in kilos.

For annual increments in weight (Fig. 2) the same sources have been used, together with my Table D (1926) for Nordic males.

The dotted curves of Fig. 1 were put in free-hand to indicate the location of possible autocatalytic curves. The dotted curves of Fig. 2

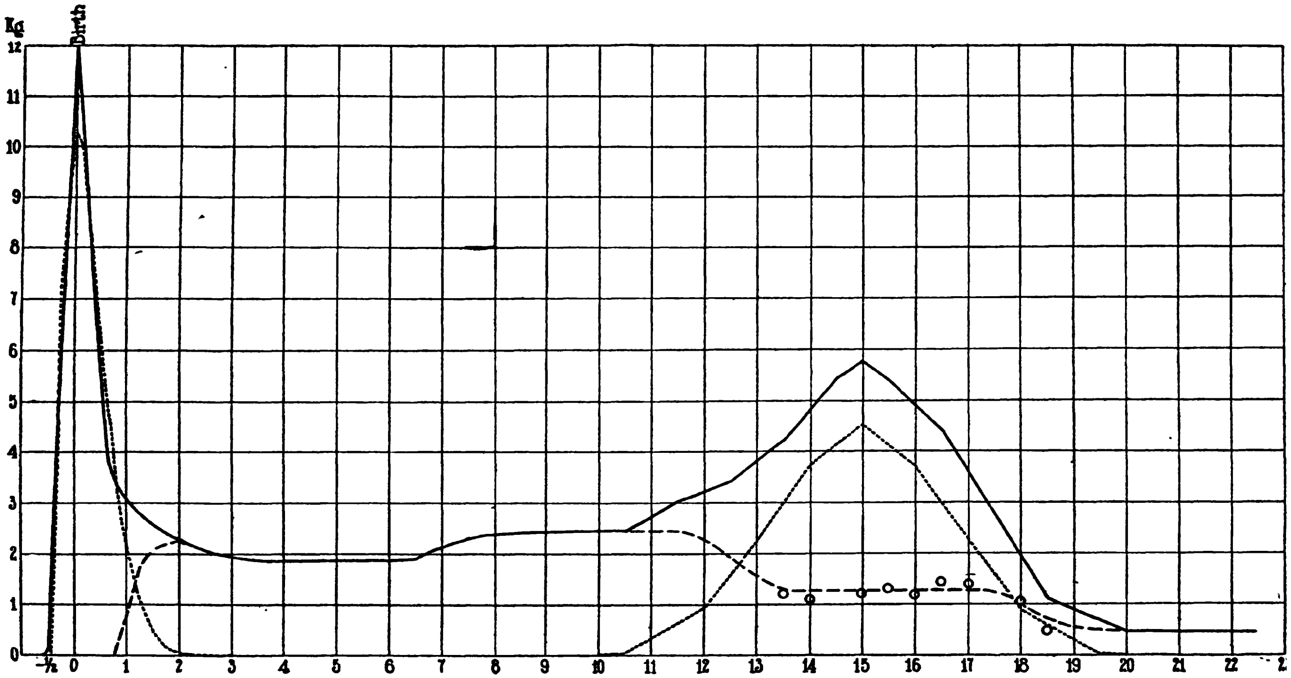

FIG. 2. Full line, the curve of annual increment rate of total body weight, male Nordic stock (see text). Dotted line to left, theoretical skew curve corresponding to increments of circumnatal growth cycle. Dotted line to right, theoretical "normal" curve of increments corresponding to adolescent growth cycle. Dash line, 0.75 to 2.25 years, full line 2.25 to 10.5 years, dash line 10.5 to 20 years, and full line beyond 20 years indicates the residual growth increments. Abscissæ, time in years; ordinates, annual rate of increments in weight in kilos. Small circles between 13 and 19 years indicate the precise points upon which is based the position of the dash line, which is smoothed between these years.

were drawn after careful computations, as described below. All statistical work was checked.

The curves are plotted on arithmetical paper, instead of logarithmic paper as is frequently done. The justification for the latter practise is found in the theoretical conception, clearly expressed by Minot (1891): "The increase of weight depends . . . . upon the amount of body substance or, in other words, of growing material present at a given time." As I pointed out many years ago (1897)[1] not all the body substance is "growing substance." During early development much water is imbibed which adds to the weight of the body, and although it may accelerate growth is not itself "growing material." During later development "body substance" is being laid down as formed substance that has primarily a mechanical or sustentative function and is not growing material. Indeed, a consideration of the complex processes of growth leads to the conclusion that to plot growth on logarithmic paper leads to just as great a distortion of the facts as to plot it on arithmetical paper. Since the latter method of plotting has the merit of simplicity, I am adopting it in this paper and suggest its uniform adoption until the advantages of some other method of plotting growth curves shall have been demonstrated.

3. RESULTS.

The arithmetical curve of growth, as plotted in Fig. 1, begins near 0 kilos at the time of fertilization of the egg. It increases slowly at first, then with ever accelerating velocity, until at birth it is shooting upward at its steepest angle. After birth the angle of slope gradually diminishes to the age of 2 or 3 years. It runs upward at a tolerably uniform rate until 7 or 8 years of age, then begins to rise more rapidly again; reaches a second maximum of slope at 14 or 15 years and then gradually approaches the horizontal.

If one contemplates this curve of growth one is struck by the resemblance of its two ends to the autocatalytic curve, to whose importance for growth Robertson has so forcefully called the attention of biologists. I have drawn in, free-hand by dotted lines, such approximate autocatalytic curves. But the growth curve, as a whole, is very far from being merely one, two, or three autocatalytic curves. The analysis of the curve may be made more readily if we transform it into a curve of growth velocities, and this is done in Fig. 2, which also is drawn on the arithmetical scale. The curve thus drawn is an instructive one. Starting at the zero base line the velocity of increments in weight rises, at first slowly, them more and more quickly, to a striking peak which corresponds with the moment of birth. At this time the child is increasing at the rate of 12 kilos per annum. The absolute velocity of growth at this period is greater than at any other time during life.

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[1] Davenport (1897), pp. 82 and 83.

After birth the velocity of growth proceeds to diminish just as rapidly as it had increased before birth. This leaves out of account the cessation of growth that is well known to occur during the 2 or 3 days after birth, since to consider it would unnecessarily complicate the main result. The curve of velocity of growth in weight runs nearly on a level from 3½ to 6½ years. It then rises very gradually for a year or two, remains constant from 8½ to about 10 years, and then proceeds to climb up to a second peak which it reaches at 15 years (in the male), at which time there is an annual growth increment of about 5.75 kilos per annum. After this peak is reached the velocity of growth diminishes to 20 years and then continues at an annual rate of slightly less than 0.5 kilos to middle life. The curve does not reach zero, on the average, because the population of adult males in the United States gains about 1 pound a year from 22 to 26 years and ½ pound thereafter until about 45 years and about ⅓ pound from then until 55 years of age.

Our velocity curve brings out clearly the fact that growth is not one autocatalytic process. It suggests the hypothesis that there are two autocatalytic cycles; one that we may call the circumnatal and the other the adolescent. The circumnatal cycle begins at fertilization of the egg; reaches a maximum at birth and probably ends at between 2 and 3 years. An attempt to fit a theoretic curve to this cycle has been made. The best fit is given by a skew curve of Pearson's (1895) Type I. Its formula is:

$$y = 854.9\left(1 + \frac{x}{5.221}\right)^{0.985}\left(1 - \frac{x}{38.876}\right)^{7.334}$$

This curve is plotted by a dotted line in Fig. 2. Its standard deviation is only 5.17 months. The y_0 value is 854.9 gm. per month or 10.259 kilos per year. This falls short of the empirical value of 12 kilos per annum partly because smoothed values were used in computing the theoretical curve. The modal velocity of 12 kilos at birth is based on Zangemeister's data (1911), which are at least conservative in respect to velocity of growth at birth.

The circumnatal curve of velocity is, as stated, a skew curve of Pearson's Type I. The index of skewness is 0.31. In the formula given above the denominator of the fraction in the second factor gives the range in months of that part of the curve that lies between birth and conception. It is 5.22 months. The denominator of the fraction in the third factor gives the range in months of that part of the curve that lies between birth and the end of the circumnatal growth spurt, amounting to 38.88 months. The theoretical range to the left of the mode is thus only 5.2 months, while, actually, development begins at 9 months before birth. However, at the end of the 6th month before birth increments in weight are only just becoming considerable (10 gm. monthly) so that the calculated curve agrees here fairly well with the observed curve. The other end of the curve is at about 39 months, at which age the observed curve of increments has nearly reached the bottom of its first peak. Thus the theoretical and observed curves are in close agreement.

The adolescent spurt is probably measured by a normal frequency polygon, whose mode at 15 years in the male corresponds with that of the total increment curve. It seems to start at about the 10th birthday and ceases at the 20th. The adolescent episode of growth thus extends over 10 years or from 10 to 13 per cent of the full span of life.

The theoretical normal curve that most clearly accords with the observed adolescent curve is shown at the right of Fig. 2 in the dotted line. There is assumed a substratum of generalized growth which, after 12 years of age, gradually declines from 1.9 kilos per annum to 0.5 kilos. The theoretical curve is drawn in accordance with the formula

$$y = \frac{n}{\sigma\sqrt{2\pi}}\, e^{-1/2\left(\frac{x}{\sigma}\right)^2}$$

where $n = 19{,}000$ kilo-years; $\sigma = 1.70$ years, and $y_0 = 4.47$ kilos.

After subtracting the two special curves from the general curve of increments there remains a residual curve. This begins at somewhere about 9 months after birth; rises to the level of the total curve, and coincides with it during the period from 3 to 10 years. That the residual curve does not start at conception would not justify the conclusion that there is no basal growth occurring, independent of the circumnatal spurt, but indicates only that the circumnatal spurt is of such high velocity and that of basal growth is of such low velocity that the latter is quite obscured by the former. From 3½ to 6 years growth is apparently entirely residual and it is very steady and constant, at about 1.85 kilos per annum. There is a slight rise during the 7th year of life to a new constant velocity of growth of 2.4 kilos per annum. This rate of growth continues until 11.5 years is reached at which time the adolescent spurt has already started. The basal increment now diminishes rapidly as the adolescent spurt speeds up. It remains at about the 1.225 kilo level of annual increment from 13.5 to 17.5 years of age and then falls away to the constant rate of 0.45 kilos per year which is reached at 19 years. Statistics gathered by insurance companies (Medical Actuarial Mortality Investigation (1912)) indicate that weight increases, in the male of average stature, about 1 pound a year from 20 to 26 years and then about ½ pound yearly to 45, as stated above.

4. DISCUSSION.

The early optimism as to the possibility of resolving the total growth curve of man into three "growth cycles" superimposed upon one another (Robertson (1923)[2]) has given way to the recognition of the great complexity of this growth curve. Thus Brody (1926)[3] states that "the smoothed time curve of growth in weight [presumably of mammals, in general] is sigmoid, but the point of inflection, or rather region of inflection, is not in its center but where slightly over one-third of the mature weight is reached. The growth curve of man is the only exception encountered." Now our data show two points of inflection that clearly approximate the logistic curve. One occurs at birth and one at 15 years, in the male. Our data do not reveal the S-shaped curve at "the third, fourth and fifth years" which Robertson finds (1923)[4], and we fail to find in Robertson's or Brody's papers any sufficient evidence of this third or "juvenile" cycle. (Compare my discussion of this matter (1926).[5])

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[2] Robertson (1923), p. 446.

[3] Brody (1926), p. 235.

What do the facts, as revealed in the curve of velocity (Fig. 2), show clearly? Besides the circumnatal and adolescent growth cycles there is a mass of growth of irregular velocity from 2 to 10½ years, in the male, which tails away toward 20 years but continues on to 50 years of age, or later. This residual curve does not fall into any autocatalytic cycle. The existence of growth outside of "the three growth cycles" has been recently recognized by Robertson (1926)[6] who introduces the idea and the term of "linear increment." In the mouse he conceives this to begin at about 10 weeks after birth and to increase in arithmetical fashion to 140 weeks, or later. This "linear increment" conception was forced from the fact that growth of mice "continues very slowly for long after the attainment of sexual maturity and dimensions which might readily be supposed to be 'adult' and, therefore, maximal. It is possible that in other animals also a similar linear accretion is occurring, and has escaped attention for lack of data concerning the late growth of the animals."

Now I suspect that the residual curve indicated by the dash line and, in part, by a full line in Fig. 2, corresponds to Robertson's "linear accretion," inasmuch as it continues past maturity; but in detail it is entirely different from Robertson's "linear accretion" since it is not a straight line at all.

One may propose a hypothesis as to the meaning of this residual curve. One may base it on the probability that besides the natal and adolescent growth accelerators there are other growth processes of particular organs or of the body as a whole. These constitute the substratum of growth of which the natal and the adolescent cycles are especially activated or accelerated episodes. Indeed, it is plain from such studies as Riddle (1925) has made on the growth of organs in the pigeons and which Scammon (1925, 1926, *a*, *b*) is making on the growth of organs in man, that the total growth is, as it were, the summation of growing parts or organs, each following a more or less independent law. Not until we understand the changes in weight of the different parts of the body from the beginning of development to maturity shall we be enabled to give an adequate interpretation of the growth curve. When that happens we shall first be in a position to direct and modify the form of the developmental curve.

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[4] Robertson (1923), pp. 445 and 446.

[5] Davenport (1926), pp. 210–212.

[6] Robertson (1926), pp. 469–473.

To illustrate, merely, the view of an important substratum of growth apart from the cycles I may refer to the findings in respect to the rate of development of three or four human organs in comparison with the growth of the body as a whole.

Thus Starkel and Wegrzynowski (1910) and E. Thomas (1911) find that the suprarenals grow rapidly in the fetus, attaining, at or about birth, a weight of 3 gm. After birth the weight falls, absolutely, to about 1.5 gm. at about 12 months of postnatal life. It then increases very slowly to about 3 gm. at about the end of 5 years. Thomas shows that the degeneration after birth affects, especially, the deeper layers of the cortex. Scammon (1926, *b*)[7] shows, in addition, that in the suprarenals there is no extraordinary prenatal acceleration of growth but only a postnatal involution. A similar postnatal retardation of growth-velocity occurs in the cerebellum (Scammon and Dunn (1924)).

The length of the uterus in the fetus undergoes extraordinary changes that have been worked out by Scammon (1926, *a*).[8] Thus in the 7th fetal (lunar) month the uterus begins to show an extraordinary spurt in growth, as compared with the body as a whole. At birth the length of the uterus is 35 mm. while, had the spurt not occurred, it would have been only about 23 mm. Within 3 weeks after birth the length of the uterus has fallen to 24 mm; and then increases slightly during the following 5 months. "This suggests," says Scammon, "that the growth of the uterus in the latter fetal months consists of a substrate of typical fetal growth plus a secondary growth increment, which, presumably, is due to an extra stimulus furnished by a hormone of placental or possibly ovarian origin. After birth the organ loses this secondary increment but retains that resulting from the early fetal growth rate." Again, reference may be made to the well known case of the thymus, which, according to Hammar (1921),[9] undergoes a rapid reduction of size and function as adolescence sets in at 11 to 15 years. This involution seems to be determined and controlled by the development of the gonads.

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[7] Scammon (1926, *b*), p. 809.

[8] Scammon (1926, *a*), p. 690.

The foregoing interesting studies on variations in the velocity of growth of human organs justify the conclusion that the development of weight in man is the resultant of many, more or less elementary, growth processes. When some special activator of development causes one or more organs simultaneously to increase in velocity of growth to a high degree then a marked maximum occurs in the human growth curve, and this may assume the form of the logistic curve of growth. Two of these episodes are of overwhelming importance. The great number of smaller growth operations are less outstanding, and overlap in time to such a degree as to become submerged in a nearly uniform, high and prolonged wave of growth. It is probable that some of these growth impulses affect not merely one or two organs of the body but are diffused more or less uniformly throughout the entire body. It is this substratum of the growth process which deserves special study and analysis in the future.

Finally, one is led to speculate on the nature of the activators of the two principal special growth accelerations—the circumnatal and the adolescent. There is some ground for entertaining the hypothesis that the adolescent spurt is especially activated by the secretions of the pituitary gland, or anterior lobe of the hypophysis; since preadolescent hypophyseal underactivity results in reduced growth and preadolescent hyperactivity in giant growth.

The tremendous velocity of growth in the circumnatal cycle seems to be activated by something coming into the fetus from the mother through the placenta. Hardly otherwise can we account for the fact that the growth process ceases its acceleration at just the time when the placental connection is broken.

Experiments should throw light on the nature of the special, as well as the general, growth activators at different stages of development.

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[9] Hammar (1921), p. 551.

5. SUMMARY OF CONCLUSIONS.

The human growth curve shows two (and only two) outstanding periods of accelerated growth—the circumnatal and the adolescent.

The circumnatal growth cycle attains great velocity, which reaches a maximum at the time of birth. The curve of this cycle is best fitted by a theoretical skew curve of Pearson's Type I. It has a theoretical range of 44 months and a standard deviation of 5.17 months. The modal velocity is 10.2 kilos per year.

The adolescent growth cycle has less maximum velocity and greater range in time than the circumnatal cycle. The best fitting theoretical curve is a normal frequency curve ranging over about 10 years with a standard deviation of about 21 months and a modal velocity of 4.5 kilos per year.

The two great growth accelerations are superimposed on a residual curve of growth which measures a substratum of growth out of which the accelerations arise. This probably extends from conception to 55 years, on the average. It is characterized by low velocity, averaging about 2 kilos per year from 2 to 12 years. It is interpreted as due to many growth operations coincident or closely blending in time.

Our curve shows no third marked period of acceleration at between the 3rd and 6th years.

The total growth in weight of the body is the sum of the weight of its constituent organs. In some cases these keep pace with the growth of the body as a whole; great accelerations of body growth are due to great accelerations in growth of the constituent organs. In other cases one of the organs of the body (like the thymus gland) may undergo a change in weight that is not in harmony with that of the body as a whole.

The development of the weight in man is the resultant of many more or less elementary growth processes. These result in two special episodes of growth and numerous smaller, blending, growth operations.

Hypotheses are suggested as to the basis of the special growth accelerations.

I take this occasion to acknowledge the valuable assistance of Miss Mary T. Scudder in the calculation of the two theoretical curves of Fig. 2.

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THE EFFECT OF ENZYME PURITY ON THE KINETICS OF TRYPTIC HYDROLYSIS.

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(Accepted for publication, August 28, 1926.)

INTRODUCTION.

In the course of an investigation of the function of pancreatic enzymes in the tannery process known as bating, the author has had occasion to study the rate of digestion of keratose by trypsin.[1] This study has resulted in the accumulation of a considerable mass of data bearing on the kinetics of the reaction under different experimental conditions. Examination of these data from the point of view of the law of mass action has brought out an interesting relation between the purity of the enzyme specimen employed and the apparent order of the reaction. It appears from this work that the less refined the enzyme, the more closely does the digestion follow the course of a monomolecular reaction. The evidence for this finding, and its bearing on the mechanism of the inactivation of trypsin, form the subject matter of this paper.

Keratose.—Since this material has not previously been studied as a substrate for enzyme action, a brief description is called for. Keratose (to apply a general term to what may or may not be a group of substances) is the first product of the alkaline hydrolysis of keratin. In its physical chemistry, keratose resembles casein, being soluble in dilute acid or alkali, but insoluble at its isoelectric point, which has been found to lie at pH = 4.1.[1] The method used in this laboratory for preparing keratose[2] consists of dissolving clean calf hair in dilute sodium hydroxide, neutralizing to pH = 8.0, filtering off any undecomposed hair, precipitating keratose in the filtrate at its isoelectric point, and washing repeatedly by decantation. The white, curdy precipitate so obtained is redissolved in dilute NaOH, and brought to pH = 8.0, which has been found to be the pH value at which tryptic hydrolysis of keratose is most rapid.[1]

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[1] Wilson, J. A., and Merrill, H. B., *Ind. and Eng. Chem.*, 1926, xviii, 185.

[2] Wilson, J. A., and Merrill, H. B., *J. Am. Leather Chem. Assn.*, 1926, xxi, 2, 50.

Enzymes.—The enzymes used in this work were commercial samples submitted to this laboratory for test as possible bating materials. They were used without any purification. The samples varied in strength from a U. S. P. pancreatin, the activity of which, measured on casein, was 7 Fuld-Gross units, to a purified trypsin of 333 Fuld-Gross units. Out of all the samples examined, three, representing respectively a very weak, a moderately strong, and a very strong preparation, were employed for the work covered by this paper. The characteristics of these samples were as follows:

Sample No.	Activity measured on	
	Casein (Fuld-Gross units).	Keratose[2] (Wilson-Merrill method).
9	333	133
6	83	27.3
2	7	4.2

EXPERIMENTAL.

Method.

The experimental method employed in studying the rate of digestion of keratose by enzymes is very similar to that used by Northrop[3] in his work with casein. The method is based upon the fact that keratose, like casein, is insoluble at its isoelectric point; while its products of digestion are soluble. Starting with a known quantity of keratose, the fraction remaining undecomposed at the end of any given time may be determined gravimetrically, and the quantity of keratose digested determined by difference. This method has the great advantage, as pointed out by Northrop, that only the first step of the digestion is studied.

The stock solution of keratose is analyzed by precipitating a measured volume at pH = 4.1, filtering through tared filter paper, drying at 100°C., and weighing. From the analytical results, the volume of stock solution containing exactly 2.000 gm. keratose is calculated. This volume is placed in a liter flask with 100 cc. of the powerful citrate-phosphate-borate buffer solution (pH = 8.0) described by Northrop, and made up nearly to 1 liter. The flask is placed in the thermostat and allowed to come to the desired temperature; then the enzyme, dissolved in a little water, is added, the solution is made up to 1 liter, and well shaken. 100 cc. aliquots are removed immediately after adding the enzyme, and at suitable time intervals thereafter. The undigested kerătose is precipitated at pH = 4.1 by running each aliquot into 50 cc. of a sodium acetate-acetic acid buffer, pH = 3.6, N/2 in acetate ion. The precipitate is allowed to settle, filtered through a tared paper, washed four times with very dilute HCl (pH = 4.1), dried, and weighed. The difference between the initial weight of keratose and that obtained after any given time interval gives the weight of keratose digested in the interval.

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[3] Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 264.

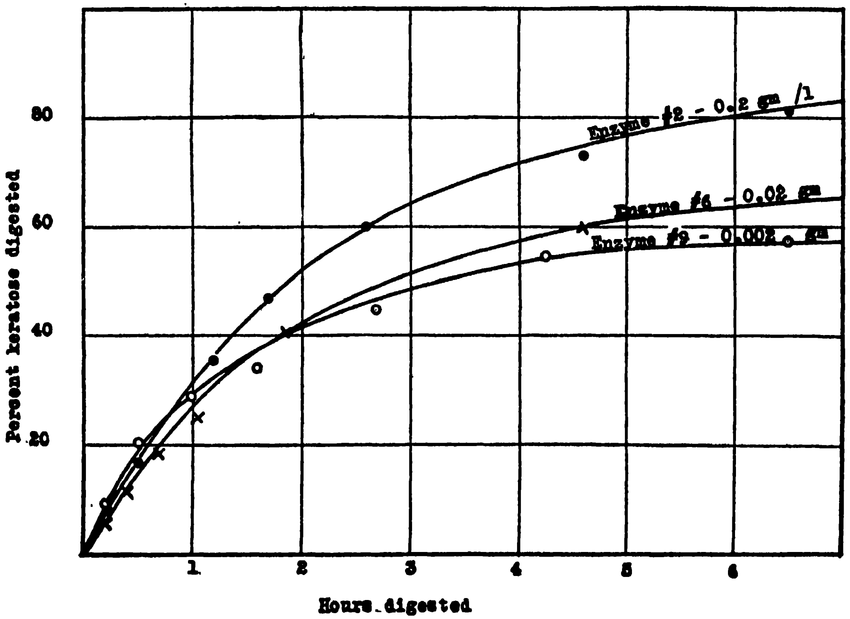

FIG. 1. Rate of digestion of keratose with different enzymes. 2.0 gm. keratose per liter, pH = 8.0, T = 35°C.

Suitable blanks were run, and corrections made for insoluble matter added with the enzyme, and for keratose digested in the absence of the enzyme. This last correction is negligible at the temperature employed for these experiments.

The temperature employed was 35°C., ± 0.05°.

Tests showed that the buffer employed kept the pH value constant to within 0.1 pH unit during the course of the digestion.

Bacterial action was inhibited by the use of thymol.

Calculations.—The per cent of the total keratose digested in different time periods was plotted against time for each series, and a smooth curve drawn through the points. Fig. 1 shows a set of such curves, obtained with the three enzymes employed in this work. It is seen that most of the points lie on or close to the curves, indicating that the percentage error is small for work of this type.

The percentages digested at appropriate time periods were read off the smoothed-out curves, and employed in the calculations.

Order of Reaction.—By the method outlined above, the rate of digestion of keratose was determined, using the three enzyme specimens under investigation. The quantity of enzyme added was so adjusted that digestion took place at approximately the same rate in all three cases. The experimental data was plotted (Fig. 1), and values for the per cent keratose digested were read off at appropriate time intervals. The velocity constant of the reaction was calculated, using the equation for a monomolecular reaction

TABLE I.

Variations of the Velocity Constant with the Stage of the Reaction with Different Enzyme Specimens.

$k_1 = 1/t \log [a/(a - x)]$

$a = 1$, x = fraction of a digested in t hours, temperature = 35°C.

Enzyme No. 2—0.2 gm. per liter.

t	0.27	0.50	1.2	1.7	2.6			
$a - x$	0.920	0.832	0.646	0.532	0.401			
$k_1 \times 10$	(1.34)	1.60	1.58	1.61	1.53			

Enzyme No. 6—0.02 gm. per liter.

t	0.3	0.7	1.2	2.0	3.0	4.6		
$a - x$	0.910	0.810	0.720	0.610	0.510	0.400		
$k_1 \times 10$	1.36	1.31	1.19	1.07	0.97	0.86		

Enzyme No. 9—0.002 gm. per liter.

t	0.2	0.5	1.0	2.0	3.0	4.0	5.0	6.0
$a - x$	0.900	0.800	0.710	0.610	0.535	0.485	0.450	0.430
$k_1 \times 10$	2.3	1.9	1.5	1.1	0.91	0.78	0.69	0.61

$$k_1 = 1/t \log [a/(a - x)]$$

placing $a = 1$ and x = the fraction of the total keratose digested in t hours. Briggsian logarithms were used.

The results of these calculations are presented in Table I, and are plotted in Fig. 2. It will be seen that for Enzyme No. 2, the weakest enzyme employed, the values obtained for k_1 are practically constant during the first 60 per cent of the reaction. In other words, the digestion does follow the course of a reaction of the first order. With No. 6, which is some 7 times as strong as No. 2, the values obtained for k_1 drop off rapidly, and with No. 9, a preparation having 30 times the activity of No. 2, the rate of decrease of k_1 is very rapid,—the "constant" falling off to about one-fourth of its initial value when the reaction is little more than half completed.

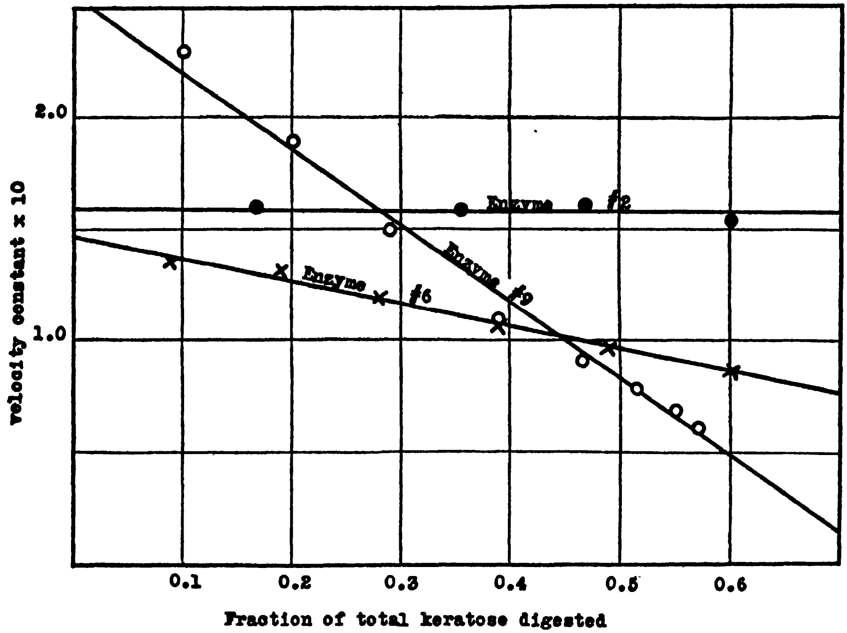

FIG. 2. Variation of velocity constants with stage of reaction for different enzymes.

In Fig. 2, k_1 is plotted as a function of the fraction of substrate digested. For the ideal reaction of the first order, the plot would be, obviously, a horizontal line. This condition is closely approached with Enzyme No. 2. With No. 6, and still more with No. 9, the departure of k_1 from constancy is sufficiently apparent.

It is significant that the plots of k_1 as a function of *fraction decomposed* are straight lines. This means that k_1 decreases in value in proportion to the amount of proteose hydrolyzed, and not in proportion to the length of time elapsed since the beginning of the reaction.

Inactivation of Trypsin.—The failure of an enzyme reaction to obey the law of mass action is commonly ascribed to progressive inactivation of the catalyst while the reaction is taking place. Two types of inactivation have been distinguished,—(1) a spontaneous, irreversible destruction of the enzyme that occurs in solution whether or not the enzyme acts on a substrate; and (2) a reversible "inhibition," due to combination between the enzyme and the products of the reaction. Northrop[4] has shown that an equilibrium, governed by the law of mass action, is set up between free trypsin and "inhibitor," on the one hand, and the complex "trypsin-inhibitor" on the other. Only the free trypsin can undergo spontaneous inactivation. The author's findings may be explained on very similar grounds.

We may assume that, in the solid state, trypsin exists in combination with some inert substance. Let us further assume that this combination, which we shall designate by the formula EnIn, dissociates in solution in a manner analogous to the dissociation of a weak acid or base. The amount of active enzyme, En, existing at any time will then be fixed by the relation

$$[\mathrm{En}] = [\mathrm{EnIn}]/(k_d\,[\mathrm{In}])$$

where k_d is the dissociation constant, and the bracketted symbols indicate concentrations.

Let us further assume that during purification of an enzyme, the concentration of active enzyme with respect to the inactive carrier is greatly increased. In a very impure preparation, [In] will be large, and [En] correspondingly small. The preparation will then be one of low activity. On the other hand, the undissociated complex, EnIn, will serve as a reservoir for En, more active enzyme being liberated as that present initially is used up by inactivation or combination with the products of the reaction. Thus [En] will remain practically constant during the course of the digestion. These are the conditions which exist when a very weak enzyme, such as No. 2, is used for protein digestion, and, as we have seen, the constancy of enzyme concentration during the course of the reaction is reflected in the closeness with which the hydrolysis follows the course of a first order reaction.

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[4] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 227, 245, 261.

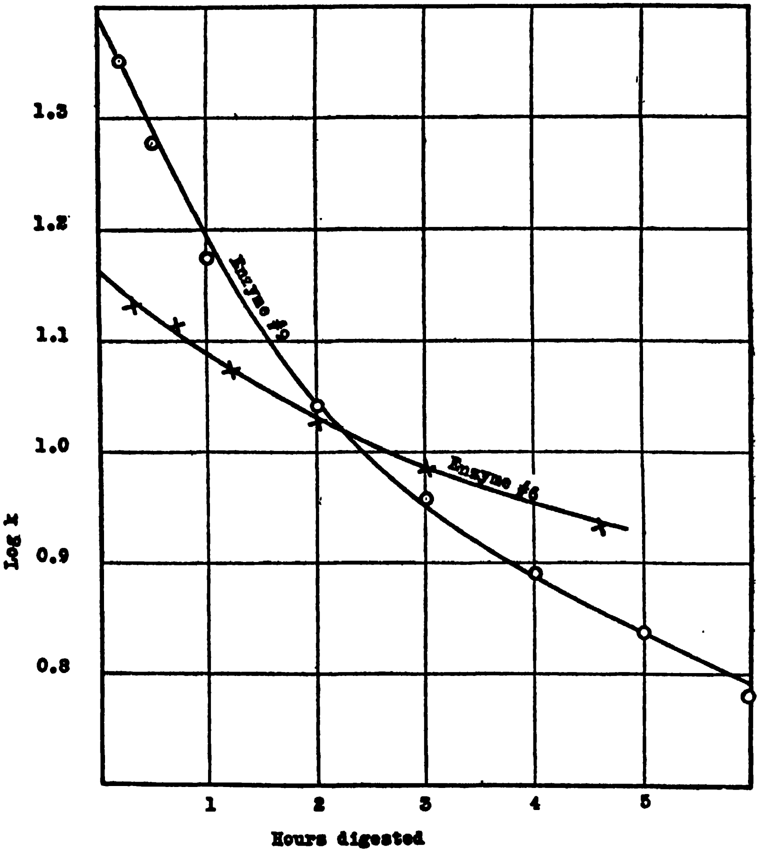

Fig. 3. Log k as a function of time.

In a highly purified enzyme, where the concentration of En is high with reference to the inert material, the complex EnIn will be largely dissociated at the start, and the preparation will manifest high activity. The free enzyme will, however, be inactivated or inhibited rapidly, and since no sufficient reservoir of combined enzyme exists, the concentration of active enzyme will diminish. Under such conditions, the rate of hydrolysis of the substrate must fall off more rapidly than would be predicted from the equation for a first order reaction. Such is the case with Enzyme No. 9.

The manner in which the velocity constant of the reaction falls off affords information as to the type of inactivation which the enzyme is undergoing. Northrop has shown that the spontaneous inactivation of trypsin follows the course of the monomolecular reaction. If k_1 is proportional to the quantity of active enzyme present, then (if the enzyme is decomposing spontaneously) log k_1 should be proportional to t, and the plot of log k_1 against t should be a straight line. In Fig. 3, log k_1 has been plotted against t for the data obtained with Enzymes Nos. 6 and 9. It is plain that k_1 does not fall off according to the equation for first order reactions. In the reversible inhibition of trypsin by combination with the products of digestion, the amount of enzyme inhibited is proportional to the fraction of substrate decomposed, and therefore k_1 will be inversely proportional to x. That this is true in the author's experiments is shown by the fact that the graphs of k_1 against x are straight lines (Fig. 2). This indicates that in these experiments the spontaneous inactivation of the enzyme is negligible, and that we are dealing chiefly with the reversible inhibition due to combination with the reaction products.

It is of interest to note that if the rate of inactivation of the enzyme happens to be the same as the rate of decomposition of the substrate, the course of the main reaction will apparently be that of a bimolecular reaction. This happens to be the case with Enzyme No. 6. The following values for the bimolecular reaction velocity constant, k_2, were calculated from the familiar formula

$$k_2 = [1/t]\,[x/(a\,[a - x])]$$

from the data given in Fig. 1.

t	0.3	0.7	1.2	2.0	3.0	4.6
$a - x$	0.91	0.81	0.72	0.61	0.51	0.40
$k_2 \times 10$	3.3	3.3	3.2	3.2	3.2	3.3

With Enzyme No. 2, the corresponding values for k_2 increase as the hydrolysis proceeds, while with No. 9 they fall off, indicating in the latter case that the enzyme is being used up at a relatively greater rate than the substrate.

SUMMARY.

The rates of digestion of keratose have been determined with three commercial enzymes, ranging widely in strength. It has been found that the weaker the enzyme preparation, the more nearly does the course of the hydrolysis conform to that of a reaction of the first order. This has been explained on the assumption that in solution an equilibrium exists between active enzyme, and enzyme combined with inert material. In very impure enzyme preparations, the large quantities of combined enzyme act as a reservoir for active enzyme, maintaining a constant concentration of active enzyme during the course of the digestion.

The author wishes to acknowledge his indebtedness to Mr. John Arthur Wilson for many helpful suggestions during the preparation of this paper, and for permission to publish.

TEMPERATURE AND FREQUENCY OF HEART BEAT IN THE COCKROACH.

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I.

It has been found that the relation existing between temperature and the frequency of rhythmic neuromuscular activity, especially when derived from many careful measurements made upon single animals at short intervals over a fairly wide range of temperature (Crozier and Federighi, 1924–25), may throw light on the question of the identity of the chemical reactions supposed to control homologous biological processes (Crozier, 1925–26, *b*). The present paper reports experiments performed further to test this hypothesis by determining the temperature characteristic for the frequency of the heart beat in *Blatta orientalis* L. Observations were made from large nymphs[1] of this species. All were secured in one lot from Birmingham, Alabama, early in 1926, and were kept at room temperature, with sufficient air, in a wire cage, where food (such as raw potato, butter, breadstuffs, and dead cockroaches) was available.

The data obtained possess added value in that the animals providing them remained whole and uninjured during the experiment. A 1000 watt lamp in a stereopticon lantern proved adequate to reveal clearly the pulsations of the dorsal blood vessel even in very darkly pigmented specimens, as viewed by the transmitted light with a low-power binocular microscope. (The absence of wings in the nymphs was favorable to this procedure.) The animal under observation, 22–26 mm. in length, was held in a glass tube, 30 cm. long and of such diameter as to press slightly upon the lateral edges of the terga. Wire gauze plugs, surfaced with cotton and set in place by wires attached to them, served to prevent movement forward or backward in the tube. The latter was connected at one end by way of rubber tubing to a suction pump, and at the other opened indirectly to the air of the laboratory; with the enclosed cockroach it was held immersed in the water of a rectangular thermostat. The bulb of a sensitive thermometer (graduated to 0.1°C.) was enclosed in air within a length of glass tubing of the same cross-section as that of the tube holding the animal, and was held close to the latter in order to approximate the same conditions. The light from the lantern, after passing through infra-red filters, was admitted to the thermostat only through an opening in a shield covering the exposed side. Here a lens concentrated the rays upon the ventral surface of the animal. A second shield of metal, fitted to the tube and with a small opening admitting illumination only to the thoracic sterna, protected the eyes of the observer from the glare and reduced photic excitation of the animal. A larger opening in the shield on the opposite side of the tube exposed to view most of the dorsum of the cockroach. Besides thus partly shielding the animal, further unnecessary photic stimulation was avoided by switching off the lamp between sets of readings.

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[1] Since one individual of the size used assumed wings at the next moult, these were presumably in the last nymphal instar.

The time for nine complete heart beats was determined with a stop-watch. As the several chambers pulsate almost synchronously in the normal cockroach, and at least at the same rate, that thoracic or (first, second, or third) abdominal segment was observed which during a given reading seemed most favorably situated. For every temperature step in a series, at least three, generally five, and occasionally up to eleven separate counts were made with a view to compensating the variations. Thus the present material represents somewhat more than 1500 stop-watch readings. In general these refer to the animal in a quiescent state; but for some sets it was virtually impossible to obtain (as was regularly attempted) complete counts without some coincident movements of body or legs. This probably did not greatly alter the average times recorded, or more in one sense than the reverse, for the consequent increase or decrease of heart rate (generally the former, but often apparently first one and then the other) seemed to be compensated in the course of two or three counts, possibly because of a fairly rapid rhythm of fluctuation (*cf.* Crozier and Stier, 1924–25). Although movements of the alimentary tract induced no obvious change in the cardiac rhythm, I largely avoided making counts while such movements were more agitated.

The temperature was changed in steps of about 1°, rarely as great as 2°. After each such change a minimum of 10 minutes was allowed for adjustment before the next set of readings was taken. A portion of this interval passed while the air within the tube acquired the temperature of the thermostat, but during the last 5 minutes or more the mercury remained practically constant at the new level. The temperatures were read to 0.01°; in but a few exceptional cases did they vary as much as 0.2° from the first to the last of any single set of readings, and they are easily correct to ± 0.1°.

At each change of temperature (10 to 25 minutes before each set of readings), fresh air, passing first through nearly 1 m. of thin glass tubing in the thermostat, was drawn through the tube holding the cockroach. This procedure was a more than ample check upon disturbances which a change of oxygen or carbon dioxide tension might cause. In this connection I cite the remarkable case of Cockroach 10 which remained alive 29 days continuously in the tube. Its heart rate was approximately unchanged during more than 10 days, even with experimental exposure to 38.4° (once) and to 5° (twice); after each experimental series, air was drawn through the tube, but not oftener than every 8 to 48 hours.

II.

Satisfactory series of observations were completed with six individuals.[2] The data are transcribed in Figs. 1, 2, 3, and 4, where the logarithm of the frequency of the heart beat has been plotted against the reciprocal of the absolute temperature. The points represent the averages of the several readings made at each temperature step in a "run". It is apparent that within narrow limits of variation all the series describe straight lines. The relation of frequency to temperature therefore fits the Arrhenius equation $K_2/K_1 = e^{(\mu/R)\,(1/T_1 - 1/T_2)}$, in which K_1 and K_2 are proportional to velocity constants at the respective absolute temperatures T_1 and T_2, R the gas constant,

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[2] Three other individuals were previously observed, in the course of developing and testing the experimental method. They yielded data not inconsistent with those about to be discussed.

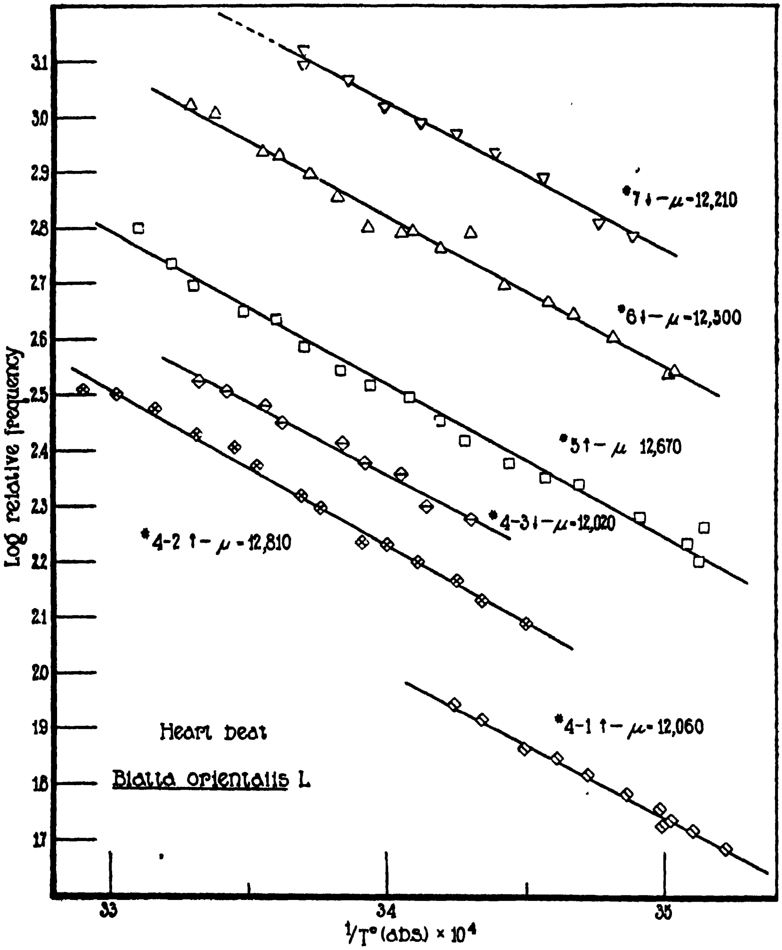

FIG. 1. Series of observations with Animals 4, 5, 6, and 7. Arrows indicate whether the temperature was raised or lowered. The frequencies are factored to raise their logarithms in each of the upper five series respectively by 1.0, 0.8, 0.6, 0.4, and 0.2 above the original values. The latter do not differ between series more than about 0.1 unit at any one temperature. The size of the symbols is such as to correspond to a probable error of ± 2 per cent of each average frequency, which is greater than the probable error found by calculation. The points marked by broken line symbols in the uppermost series were obtained before the animals had become adequately accustomed to the high temperature; hence the line was fitted to the rest of this series without considering them. Such deviations illustrate the necessity for careful thermal adaptation.

and μ the critical thermal increment or temperature characteristic (Crozier, 1924–25). The value of μ is fixed by the slope of the line best fitted to the points plotted; it may be computed from the graph, the frequencies being measures of the velocity constants.

Separate and repeated fittings to the series obtained from five individuals, shown in Figs. 1 and 2, lead to values of μ ranging from 12,020 to 12,810 calories for temperatures between 10° and 38°.[3] The weighted average μ = 12,500. Of the five animals one was cooled below 10°; two series from 19° to 5° show a "break" or marked change of temperature characteristic occurring at about 10°, such that for lower temperatures μ is of the order of 18,100 (Fig. 2).

Fig. 3 is a mass plot of the series (above 10°) which are shown in Figs. 1 and 2, but adjusted by suitable factors to coincide at $1/T$ = 0.0034. The combined series give a straight line for which μ = 12,500 calories. This represents the mean for the range 10–38°, and is subject to a probable error of ±0.6 per cent. The vertical width of the band of points, which as in other cases (Crozier and Stier, 1924–25) forms a ribbon with parallel margins, is an index of the variability encountered in the normal cockroach. On the basis of a selection of sets of readings representing wider deviations than usual in the data here reported, the latitude of variation for any one individual is with very few exceptions well within ±7 per cent of the mean frequency at any one temperature. On a like basis, the probable error of the mean frequencies entered in the graphs is found seldom to exceed ±2 per cent, being much less in about half the observations.

In contrast to the others, one individual yielded data from which a clearly different magnitude of μ must be derived. When a first "run" revealed a value of the order of 14,300, I made additional series of observations upon the same individual within 18 days after the first. Considered separately, the latter series give rise to values of μ respectively higher and lower than the initial determination, yet safely

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[3] A very slightly lower average value is given by the "runs" of falling temperatures than by those of rising temperatures, but the difference is not great or consistent enough to be significant. Thus, of six comparable series (Fig. 1) the three during which the temperature was lowered give 12,020, 12,210, and 12,500 (mean = 12,240), and the other three during which the temperature was raised give 12,060, 12,670, and 12,810 (mean = 12,510).

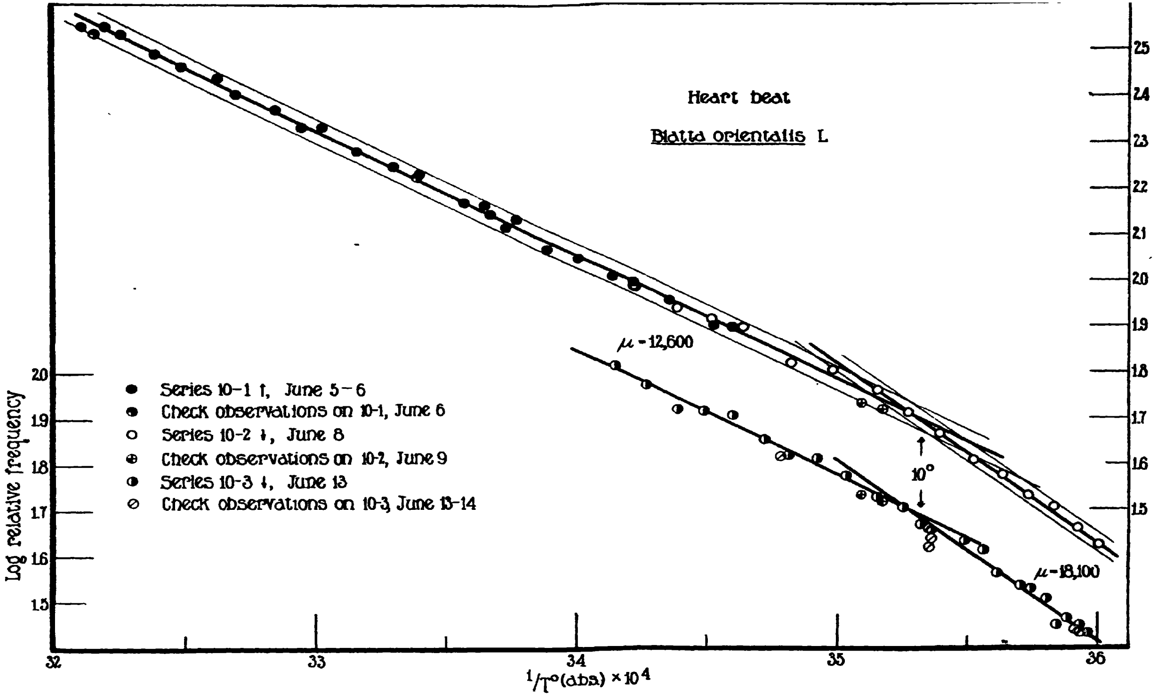

FIG. 2. Series of observations obtained with Animal 10. Separation of two "runs" is effected by plotting against ordinates at left and right, the scales differing by 0.2 of log frequency. The final (lower) series would otherwise fall only about 0.007 unit below the combined upper series. The direction of change of temperature in each series of readings is indicated by the arrows. (Since check observations on returns from extreme low temperatures revealed a lag in resumption of higher frequency, such points were not accorded full weight in fitting the lines.) It is to be observed that if one were in possession merely of data from a series of readings over the temperature range 5–19°, difficulty would be experienced in obtaining a significant μ, and the data as plotted might seem to fall upon a curve.

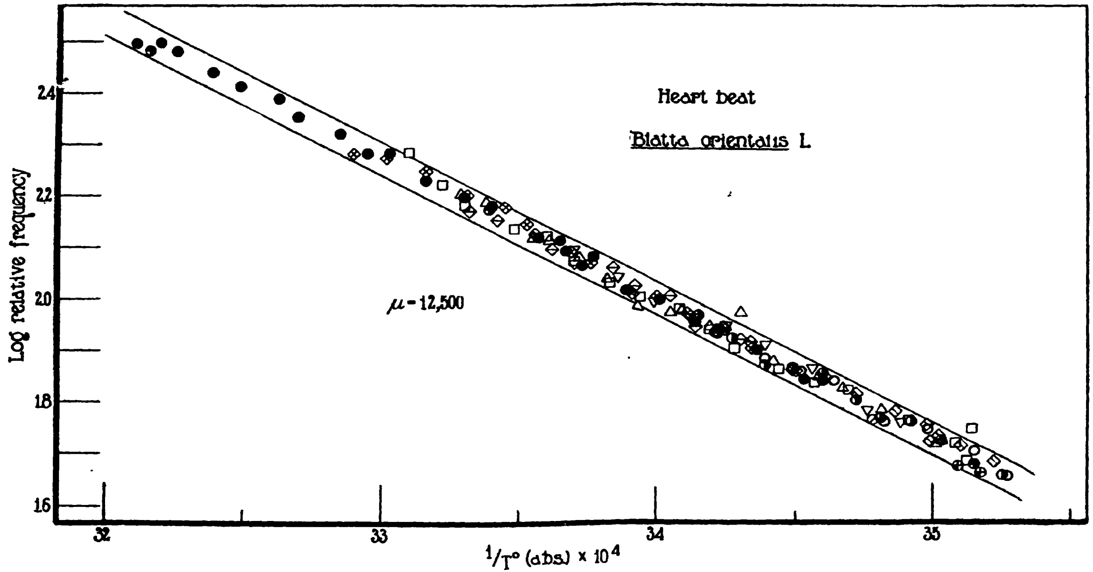

FIG. 3. Combination of series (above 10°) obtained with Animals 4, 5, 6, 7, and 10, all series being made to coincide (by suitable factors) at $1/T = 0.0034$. The symbols are the same as in Figs. 1 and 2.

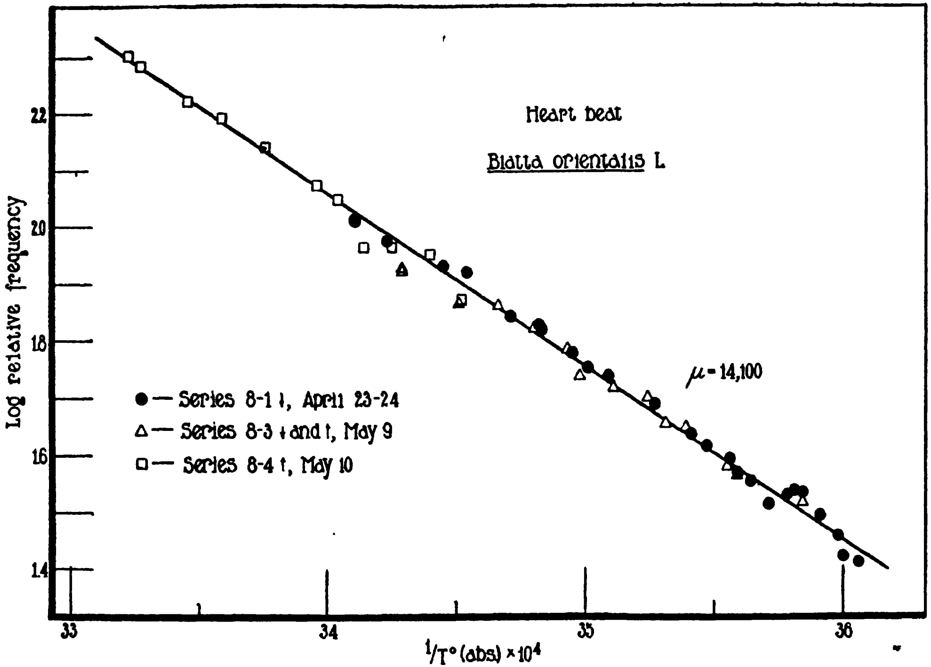

Fig. 4. Three series obtained with Animal 8 plotted to one scale. Arrows indicate whether the temperature was raised or lowered (or first one, then the other).

comparable and not to be classed with the previous cases averaging 12,500. Fig. 4 shows the data from three "runs." Coincidence is so nearly complete that a single line is drawn to represent the average, according to which $\mu = 14{,}100$ (correct to ±300 or better) over the range 4.5–28°.[4] In this case no change to a higher temperature characteristic near 10° is demonstrable for the lower rage of temperature. Excepting the first series, the latitude of variation is greater than for the other five animals (as much as double), yet the probable errors of the individual plotted frequencies only rarely exceed ± 2.5 per cent. For this animal, therefore, both a difference in μ and the absence of a critical temperature at 10° ± serve to characterize the control of its heart rhythm as different from that of the others.

III.

The results make it clear that the heart rate in the late nymphal stage of the cockroach typically varies with the temperature in a way defined by $\mu = 12{,}500 \pm$ calories; but that a considerably higher critical increment (*ca.* 18,100) probably holds for the same animals at low temperatures, the critical point (Crozier, 1924–25, 1925–26, *a*) at which the change occurs being 10°±. Although most of the animals reveal above this temperature an approach to the mean value 12,500, with a constancy indicated by a standard deviation amounting only to 2.3 per cent, an exceptional individual may be found which in some (still unknown) respect differs from the typical so that the control of the heart rhythm lies in a process whose μ is definitely of another magnitude, namely 14,100±.

The pulsation of the dorsal vessel of insects is considered to be controlled by the central nervous system (Zawarzin, 1910–11), and Carlson (1905–06) reported evidence of both augmentary and inhibi-

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[4] Another series obtained from this animal showed such abnormally high variability that μ could not be determined with any reasonable degree of accuracy. Except that the observations were in this instance begun immediately after transference to the tube, little cause can be given for this instability, but a correlation with it of a general drop in heart rate may be noted. Both occur in cases of subnormal vitality, as other observations show. Thus Cockroach 10 showed both a marked increase in latitude of variation and an absolute slowing down of the heart rhythm after 4 weeks in the experimental tube.

tory innervation of the heart of an orthopteran (*Dictyphorus*).[5] The typical value of μ (12,500) for the cockroach heart rate was therefore expected to accord quantitatively with values determined for other non-respiratory neuromuscular activities of arthropods presumed to depend upon the rate of "central nervous discharge" (Crozier, 1924–25). It does so within limits of difference ascribable to errors of curve fitting or to consequences of uncontrolled body or limb movements. Thus Crozier and Stier (1924–25) have listed a number of such phenomena, for which μ = 12,200±. More recently they have (1925–26) reported the same value applying to locomotor activity in tent caterpillars. In addition, Federighi[6] finds the heart beat of the annelid *Nereis* to show μ = 12,400.

Neither is the exceptional case where μ = 14,100± entirely without counterpart, although it is very infrequent (Crozier, 1925–26, *b*). A like value appeared (as an exception in series yielding 16,200) among Glaser's (1925–26) determinations for the heart rate of a pteropod and (as exception to 11,100) in Cole's (1925–26) findings for locomotion in *Planaria*. The data in the present instance offer nothing toward explaining the atypical value, for no difference was apparent in the treatment or condition of the animals. All that can be said is that the results point to the possible validity of 14,000+ as the temperature characteristic of one of several chemical reactions which may be supposed necessary to more than one type of vital process (Crozier, 1925–26 *a*, *b*) but which only exceptionally proceeds so slowly as to assume a governing rôle.

IV.

SUMMARY.

The frequency of pulsation of the intact heart in nymphs (final (?) instar) of *Blatta orientalis* L. increases with the temperature according to the equation of Arrhenius. The constant μ has typically the same value, within reasonable limits of error, as that (12,200) deduced for other, homologous activities of arthropods where the rate of central nervous discharge is perhaps the controlling element, namely 12,500 ± calories for temperatures 10–38°C. Below a critical temperature of about 10° a change to a higher value of the temperature characteristic occurs, such that μ = 18,100 ±. Exceptionally (one individual) μ = 14,100 ± over the whole range of observed temperature (4.5–28°).

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[5] More recently Alexandrowicz (1926) has described in detail the innervation of the cockroach heart.

[6] Federighi, H. (unpublished experiments).

The quantitative correspondence of μ for frequency of heart beat in different arthropods adds weight to the conception that this constant may be employed for the recognition of controlling processes.

It is a pleasure to acknowledge my indebtedness to Professor W. J. Crozier for his suggestion and guidance of this work.

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SOME PHYSICOCHEMICAL PROPERTIES OF DISSOCIATED SPONGE CELLS.

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(Accepted for publication, July 12, 1926.)

I. INTRODUCTION.

It has been known since Wilson's discovery (1) that dissociated cells of *Microciona* come together and form aggregates, which by further transformation develop into new sponges. Similar processes were observed in fresh water and in calcareous sponges (2, 3), and in hydroids and alcyonarians (4, 5). Recently one of us (6, 7), in studying the behavior of dissociated cells of *Microciona*, found that the formation of aggregates is due to the ameboid movement of so called archæocytes, that is, unspecialized cells of the sponge mesenchyme, which upon separation creep in various directions and coalesce with other cells of the same species which happen to lie on their route. According to these observations, aggregation is easily affected by changes in the surrounding medium. In pure isotonic solutions of NaCl or KCl the ameboid movement is entirely inhibited, and the addition of at least one of the alkaline earth metals, either Ca or Mg, is necessary to produce the aggregation of cells. The addition of acids or bases to a suspension of cells also causes significant changes in their behavior, inhibiting their movement and changing the adhesive properties of the protoplasm. In mixed suspension, the cells coalesce only with cells of their own species, forming separate aggregates; while in alkaline sea water the *Microciona* aggregates become surrounded by the *Cliona* cells.

The present investigation is an attempt to deal in a quantitative manner with the equilibrium relations involved between the cells of two siliceous sponges, *Microciona prolifera* Ver. and *Cliona celata* Gr., and acid or base.

As a preliminary to the account of the investigation of the sponge cells, we report a titration of 0.00280 molar NaAc in 0.520 molar NaCl solution; this solution served as the medium in experiments with the cells.

II. The Hydrogen Ion Activity in a Solution Containing 0.00280 Mols of NaAc, 0.520 mols of NaCl per Liter, and Various Amounts of HCl and NaOH.

A medium for the titration of the cells of the sponges must answer several requirements. First of all, it must be isotonic with the cells; secondly, it must be of such a nature as to prevent aggregation of the cells (7); and, thirdly, it must have a certain buffer value on the acid side of neutrality (since most of the observations were made in that range) so as to yield reproducible E. M. F. measurements.

After several trials we found that a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl, and varied amounts of HCl and NaOH, answered practically all of our requirements. It gives fairly reproducible E.M.F. measurements, but its buffer value is not large enough to mask the effect of the acid or base bound by the sponge cells.

Several investigations were made on acetate buffers containing different amounts of NaCl. L. Michaelis and R. Krüger (8) studied the hydrogen ion activity of a 0.02 N mixture of equal amounts of NaAc and HAc in the different salts. They found that in 1 molar NaCl the mixture has a pH of 4.484, the pK′ evidently being equal to 4.484.

L. Michaelis and K. Kakinuma (9) in their contribution to the electrochemical measurements of the activity of ions found that 0.01 molar solutions of equal amounts of NaAc and HAc, containing different amounts of NaCl, have different hydrogen ion activities. A solution containing 0.1 mol of NaCl had a pH equal to 4.607, a 0.5 molar solution a pH of 4.503, and a 1.0 molar solution a pH of 4.448.

G. S. Walpole (10) investigated the pH of a mixture of HAc, NaAc, and NaCl. The concentration of Ac in this system was 0.20 N; a 1:1 mixture gave a pH of 4.58. The measurements were made at 18°C.

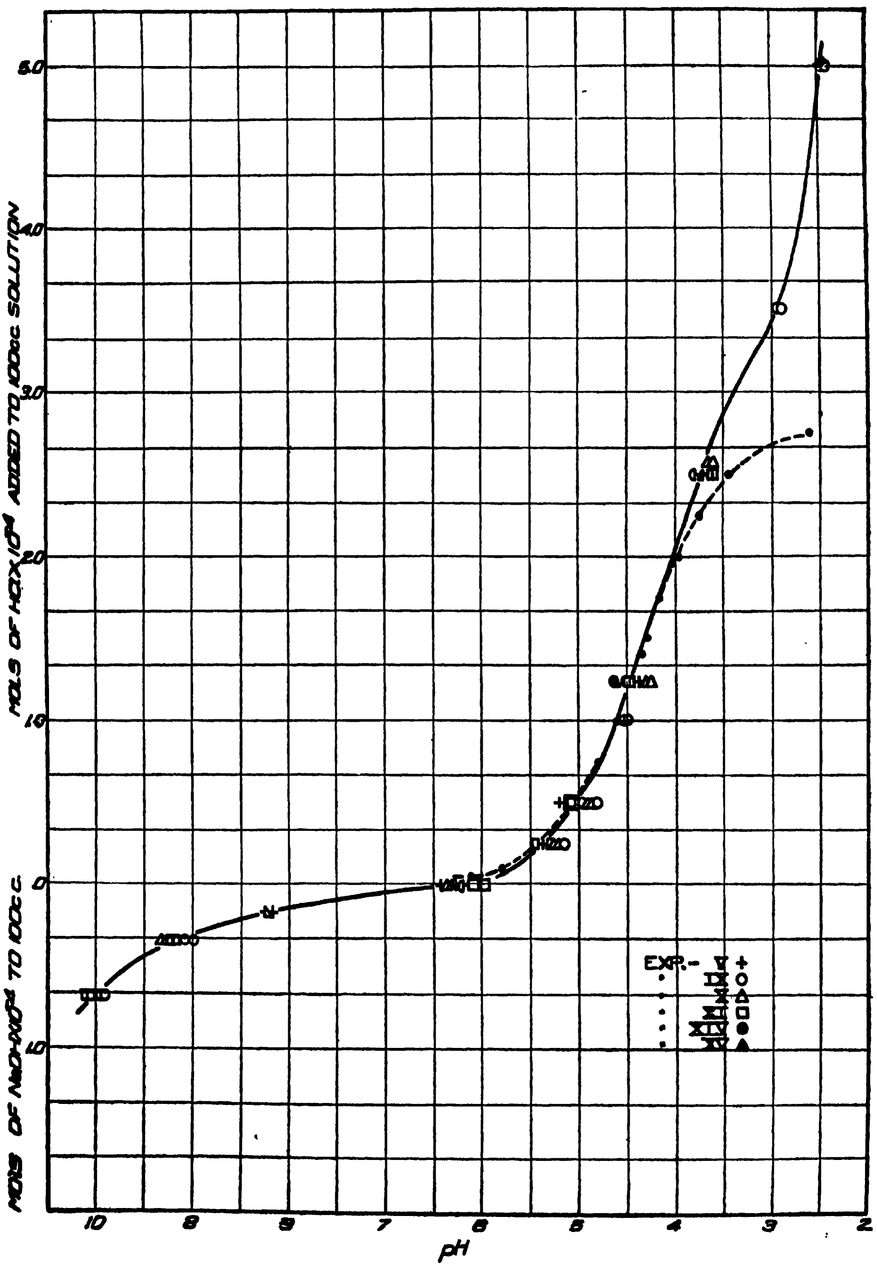

FIG. 1. The hydrogen ion activity in a solution containing 0.00280 mols NaAc, 0.520 mols NaCl per liter, and varied amounts of HCl or NaOH.

J. N. Brönsted (11) recalculated the experimental data obtained by Walpole in terms of fundamental thermodynamic functions.

The results of our investigation are graphically represented in Fig. 1.

All the pH measurements reported were made by means of a Leeds and Northrup potentiometer. The E.M.F. of the hydrogen electrode was measured against a 0.1 N KCl calomel electrode, using a saturated KCl bridge. The pH's reported were recalculated by the equation:

$$\text{pH} = (\text{E.M.F. observed} - \text{E.M.F. calomel}) \; 1 \div 0.001983$$

For the E.M.F. of the calomel electrode we used the value given by Lewis and Randall (12). No correction for the diffusion potential was made.

Our experiments were carried out at slightly different temperatures. The effect of temperature on the activity is not a negligible one. We corrected for the influence of temperature by interpolating between experimental points. Fig. 1 represents the titration of our acetate buffer at about 21–22°C.

In this figure the dotted line represents the pH values calculated by the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pK}' + \log [(\text{NaAc}) \div (\text{HAc})]$$

It is evident from Fig. 1 that the equation holds over a considerable range. It fails, however, to describe the experimental data in the range where the amount of NaAc becomes very small. The average pK′ for acetic acid in our system is about 4.37.

III. The Hydrogen Ion Activity in a Suspension of Cells of Microciona prolifera or Cliona celata, in Which the Amount of Acid or Base is Varied, but the Concentration of Cells Is Kept Constant.

In its natural habitat *Microciona* is usually found attached to rocks or shells, frequently occurring on oyster beds. The sulfur sponge, *Cliona*, is a boring sponge; it infests the shells of various pelecypods (both living and dead), and having bored through them, grows farther, reaching an enormous size.

The sponges used in the experiments were collected in the vicinity of the Woods Hole laboratory. *Microciona* was taken from the mouth of Wareham Bay, and from Waquoit Pond near Falmouth, Massachusetts. *Cliona* was obtained in Squeteague Harbor near North Falmouth, Massachusetts. Both species inhabit shallow waters and normally sustain considerable fluctuations in salinity. In Waquoit Pond and in Wareham River the salinity, according to our observations, varies with each tide from 27 gm. per liter at high tide to 16 at low tide.

Though the salinity in the laboratory tanks at Woods Hole is much higher, varying from 31 to 32, no harmful effect was noticed, and the sponges sustained the new environment very well. As a rule, however, the sponges used for experimentation were not kept longer in the aquarium tanks than 5 days. Experience shows that as a result of prolonged life in the aquarium they undergo physiological and anatomical changes, and become unfit for experimental work.

The following procedure was adopted to obtain a suspension of sponge cells.

(1) Each piece of sponge was washed, all dead portions were cut away, and it was cleaned from all foreign substances, such as small pebbles, sand, mud, or algæ.

(2) The sponge was then placed for 15 minutes in a 0.520 molar NaCl solution, the solution being changed twice.

(3) The material was squeezed through bolting silk No. 20 into a solution containing 0.00280 mols NaAc and 0.520 mols NaCl per liter.

(4) The next procedure consisted in centrifuging and washing twice with a solution of the same concentration of salts as that described in (3).

(5) The suspension of cells thus obtained was transferred to a vessel through which a steady current of CO_2-free air was bubbled. This last process was necessary in order to free the solution from any small amounts of bound or free carbon dioxide which might possibly be present, and also to keep the cells from settling to the bottom of the vessel. By bubbling air through the suspension, it can be kept for 24 hours without sedimentation and aggregation of cells.

The suspension of sponge cells obtained consists of archæocytes, collencytes, pinacocytes, desmacytes, and choanocytes. The percentage composition of the suspension may be given as follows: *Micro-*

ciona; 25.5 per cent archæocytes, 9.9 per cent collencytes, and 64.6 per cent desmacytes, pinacocytes, and choanocytes; *Cliona*; 15.4 per cent archæocytes, 15.0 per cent collencytes, 69.0 per cent desmacytes, pinacocytes, and choanocytes.

During the preparation of the suspension the cells were subjected to rather vigorous mechanical treatment. Part of them might have been cytolyzed. The products of this cytolysis might appear in the watery phase, and might be responsible for the binding of any acid or base added to the system.

TABLE I.

Effect of the Number of Washings on the pH of a Suspension of Cells of Microciona prolifera.

Experiment *VI. Washing* solution contains 0.520 mols of NaCl, 0.00280 mols *of NaAc,* and *1.25* $\times 10^{-3}$ mols of HCl per liter. 30 minute intervals between consecutive washings.

No. of washings. (1)	E.M.F. (2)	Temperature. (3)	pH (4)
	volts	°C.	
2	0.6230	23.8	4.88
2	0.6218	23.8	4.86
3	0.6274	23.8	4.95
3	0.6272	23.8	4.95
4	0.6251	24.2	4.91
4	0.6247	24.3	4.90
5	0.6235	24.2	4.88
5	0.6233	24.2	4.88
6	0.6205	24.1	4.83
6	0.6206	24.1	4.83
NaCl solution used in this experiment.	0.5952 0.5960	24.7 24.7	4.40 4.41

In order to determine whether we were dealing in our experiments with the acid- or base-binding property of the cells, or with the effect of some unknown product of cytolysis, we carried out the experiment reported in Table I. In this experiment a suspension, prepared in the way already described, was further washed with a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl per liter. After each washing the suspension was centrifuged, and the pH of the supernatant liquid determined. It is evident that the pH of the suspension remains practically constant. If the acid-binding property of this system was dependent upon the product of cytolysis, the pH should gradually have risen until it reached the pH of the washing solution. No such phenomenon was observed. We must conclude, therefore, that in this case we are dealing with a property very closely associated with the living cells.

In our experiment we added acid or base to the suspension of the sponge cells. When any acid or base is added to a system containing basic or acid radicals, a displacement of the equilibrium occurs. The establishment of the new equilibrium takes a certain length of time, depending upon the properties of the system. To test the effect of time on the system containing a rather large amount of acid and a suspension of cells of *Microciona prolifera,* we carried out the experiment reported in Table II. As is seen from the table, the pH values are almost constant. The difference in the pH value of the cell suspen-

TABLE II.

Effect of Time upon the Establishment of an Equilibrium between the Cells and the Acid Added.

Solution: 0.520 mols NaCl and 0.00280 mols NaAc per liter.

Suspension: 51.9 × 10^8 cells of *Microciona* suspended in 100 cc. of solution; 1.25 × 10^{-4} mols HCl added to it.

Time elapsing between the addition of the acid and the E.M.F. measurement. (1)	E.M.F. (2)	Temperature. (3)	pH (4)
min.	*volts*	°*C.*	
48	0.6219	21.8	4.892
	0.6218	21.8	4.890
63	0.6223	22.0	4.896
	0.6223	22.0	4.896
99	0.6241	22.0	4.919
	0.6240	22.0	4.918
125	0.6249	22.4	4.933
	0.6248	22.4	4.931

sion and the same solution without the cells, in this experiment, is *equal to 0.5 of a pH unit, while the difference* between the first reading and the last is only 0.04 of a pH unit. There are three possible causes of this variation. One of them has already been pointed out; namely, the time factor in the establishment of the new equilibrium. The second factor which must undoubtedly be present in any system containing living material is that of metabolism. The products of

TABLE III.

Vitality Tests of the Cells Used in the Titration Experiments.
Microciona prolifera.

Experiment No. (1)	pH of the suspension. (2)	Condition of cells after titration experiments.	
		Immediately. (3)	12 hrs. later. (4)
VIII	4.56	Normal.	Very small aggregates slightly adhering to glass; many cytolyzed cells.
V	4.82	“	Small globular aggregates adhering to glass; few cytolyzed cells.
III	5.90	“	Normal aggregates adhering to glass; few cytolyzed cells.
V	6.13	“	Normal aggregates adhering to glass.
X	6.36	“	“ “ “ “ “
X	6.96	“	“ “ “ “ “

TABLE IV.

Vitality Tests of the Cells Used in the Titration Experiments.
Cliona celata.

Experiment No. (1)	pH of the suspension. (2)	Condition of cells after titration experiments.	
		Immediately. (3)	12 hrs. later. (4)
IX	3.06	Part of cells cytolyzed.	Cells strongly adhering to glass; no aggregation.
IX	3.71	Normal.	“ “ “ “ “ “ “
IX	4.09	“	“ “ “ “ “ “ “
IX	6.59	“	Normal aggregates.
IX	6.70	“	“ “
XI	7.34	“	“ “

the metabolism might possess acid or basic properties of their own, and might gradually change the pH of the medium. Without knowing the chemical nature of these metabolic products, one cannot determine their influence upon the pH of the suspension. The third factor which may be responsible for this variation is the beginning of cytolysis. It is quite probable that in such acid solution irreversible changes occur in the cells, producing more and more titratable material. This will be seen from the discussion of the vitality tests of the cells treated with acid.

In all subsequent measurements we used 45 minutes for the equilibration time.

The next problem with which an investigator of living matter is confronted, is to determine whether or not his chemical manipulations have caused a permanent injury or death to the object of his experiment. The best test of this, is to examine the cells immediately after the experiment and to observe their behavior when they are brought back to a normal environment.

For this purpose we conducted the following tests in conjunction with the measurements of the pH which resulted from the addition of acid or base to the suspension of sponge cells: the cells used in the experiments were washed with sea water and examined under the microscope to determine whether they were alive or not, then 1 cc. of suspension was added to 9 cc. of sea water, and the mixture left undisturbed for 12 hours. This period is long enough for uninjured cells to coalesce and form globular aggregates which adhere strongly to the glass (7). The cells irreversibly affected by previous treatment are partially cytolyzed and form aggregates of irregular shape. Dead cells form loose sediment not adhering to the glass.

Tables III and IV list the results of the vitality tests carried out on cells taken from the titration experiments. It can easily be seen that the susceptibility of *Microciona prolifera* to acid solutions used in the experiments is much higher than that of *Cliona celata*. Though at the end of Experiments V and VIII the cells of the former remain alive and under the microscope appear to be normal, their ameboid movement is retarded. After 12 hours, instead of forming a few large aggregates as always happens under normal conditions, they coalesce into numerous small groups; many cells at the end of this period become cytolyzed. This occurs when the pH of the supernatant liquid of the suspension is 4.56.

The cells of *Cliona celata* endure much higher acidity remaining uninjured at pH 3.71, though their ameboid movement after such treatment is inhibited.

The difference between the cells of the two sponges can undoubtedly be correlated with the fact that for a given change in pH in fairly *acid solutions, Microciona* binds more acid than *Cliona*. We may suspect therefore that greater chemical changes occur in the first than in the second.

It must be borne in mind that, due to the rough treatment during squeezing, washing, and centrifuging, the cell suspensions may contain a certain amount of cytolyzed material; so the presence of a small number of cytolyzed cells cannot be attributed entirely to the effect of acid solution. Further increase in hydrogen ion activity will certainly cause a complete cytolysis and death of the cells. As can be concluded from the examination of Tables III and IV, the critical concentration probably lies just below pH 4.5 for *Microciona* and about 3.7 for *Cliona*. Above these values, the largest part of the cells examined under the microscope immediately after the titration experiments showed no evidence of injury.

The amount of acid or base bound in titration of a suspension depends upon the concentration of cells in that suspension. Our titration experiments were carried out with suspensions of a definite concentration. We shall express this concentration in terms of the number of cells present in 100 cc. of the suspension. This method of expressing the concentration is not strictly correct. Cells have their own volume; therefore the volume of "free" solution depends upon the number of cells present. Any computation of acid or base bound referred to 100 cc. will deviate from the true value by the volume of cells present in the system. However, we believe that by using rather dilute suspensions of cells we made this error negligibly small.

In determining the number of cells in a given suspension, the following procedure was adopted: 1 cc. of this suspension was diluted one hundred times and shaken well. 1 cc. of this suspension was transferred to a counting cell. A uniform distribution of the cells was secured by the use of a pipette. In about 10 minutes the cells settled on the bottom and could be counted with a Whipple square micrometer. We counted the cells in ten fields of view taken at random at various parts of the counting cell. From the average number obtained by this procedure the total number of cells in 1.00 cc. of suspension was estimated. The results were usually accurate within 5 per cent.

If the stock suspension was found to differ from the desired concentration, it was diluted to the appropriate extent. After the dilution, the number of cells was checked once more.

In all our experiments we titrated with HCl and NaOH, and for this reason our medium contained a large amount of NaCl. Any addition of small quantities of Cl or Na produced, therefore, a practically negligible change in the concentration of either Na or Cl. Any reaction of the cells will, therefore, be due entirely to the change in the concentration of free acid or base as measured by the hydrogen ion activity or its dependent variable, the hydroxye ion activity. This statement would be accurate if our systems had not contained NaAc. Upon the addition of an acid, however, the concentration of Ac^- decreases proportionally to the acid added. Therefore, in addition to the variables (H^+) and (OH^-) we have the variables (HAc) and (Ac^-). Evidently this second set of variables cannot be neglected. A simple way to test the influence, if any, of the concentration of HAc and Ac^- is to titrate the cells in a solution of 0.520 molar NaCl in the absence of NaAc. Such an experiment is hardly quantitative in a slightly acid solution, but in a medium containing a rather large amount of acid the E.M.F. becomes reliable. Therefore we brought a solution containing 0.520 mols of NaCl per liter to a pH of 4.50 by adding to it a known amount of HCl. Then to the same solution we added about 50×10^8 cells of *Microciona prolifera* and by the addition of HCl brought it to the same pH as the solution of NaCl. It was found necessary to add more acid to the cells than to the NaCl solution in order to make the two solutions isohydrionic. Evidently the amount of acid added to the cells minus the amount of acid added to the NaCl solution is the amount of acid bound by the cells. It was found to be equal to 1.2 ± 0.2 mols HCl $\times 10^{-4}$. If we compare this figure with the one obtained from the titration of the cells of *Microciona prolifera* in the presence of NaAc (Fig. 2) we find a complete agreement. Evidently the (HAc) and (Ac^-) are not the controlling factors in the titration in question.

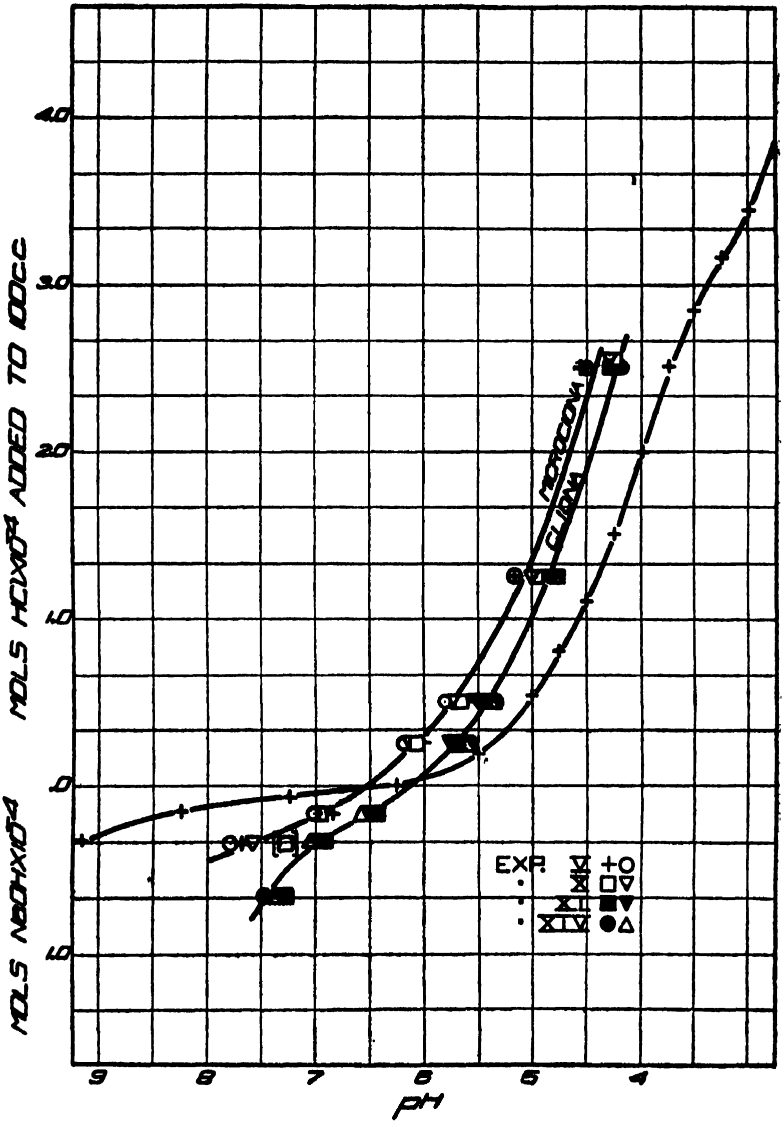

FIG. 2. The hydrogen ion activity in a solution containing NaAc and NaCl, a given number of cells of *Microciona prolifera* or *Cliona celata*, and varied amounts of HCl or NaOH.

Microciona: Experiment V, 52.4 × 10^8 cells; Experiment X, 49.3 × 10^8 cells. *Cliona:* Experiment XI, 57 × 10^8 cells; Experiment XIV, 51.1 × 10^8 cells.

The titration of the suspension was effected by adding to a known amount of cells a given amount of HCl or NaOH. The cells were then centrifuged and the pH determined electrometrically on the supernatant liquid. Two E.M.F. measurements were carried out on each sample. The results of two experiments are given in Fig. 2, together with the titration of the acetate buffer, taken from Fig. 1.

As may be noticed from the titration curve of *Microciona prolifera*, the two experiments disagree slightly with each other in the upper part of the curve. The reason for this disagreement is a difference in concentration of the cells in the two experiments. By drawing a line between the experimental points we can take care of this influence, and the line of titration would represent a titration of suspension having approximately 50.8 × 10^8 cells per 100 cc. A similar behavior is shown by the cells of *Cliona*, though to a lesser extent.

The titration curves obtained for the sponge cells are only functions related to the acid- or base-binding properties of the cells. The curves will have different shapes in media containing different buffers.

If we subtract at any pH the amount of acid necessary to bring the acetate buffer to that pH from the amount of acid added to the cells to bring them to the same pH, we shall obtain a value characteristic of the suspension—the amount bound by the suspension.[1]

Such a calculation was made for both *Microciona* and *Cliona* for the slightly acid and basic ranges of the titration curve. The results of the estimates are given in Fig. 3. They are probably accurate within about 8 per cent.

The function thus obtained is of fundamental importance for the estimation of the physicochemical properties of cells. Each curve has

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[1] This procedure is not strictly correct since the free base or acid is related to the hydrogen ion activity by the equations:

$$(\mathrm{HCl}) = \gamma_1 (a_{\mathrm{H}^+})$$

$$(\mathrm{NaOH}) = \gamma_2 K_w \div (a_{\mathrm{H}^+})$$

in which the activity coefficients γ_1 and γ_2 vary with the change in concentration of HCl and NaOH.

But, since our system contains a large amount of NaCl, the change in the activity coefficients between the acetate titration curve and the one of the sponge cells is probably small.

two parts; one above the zero point where the sponge behaves as a base, and one below where it behaves as an acid. The zero point,

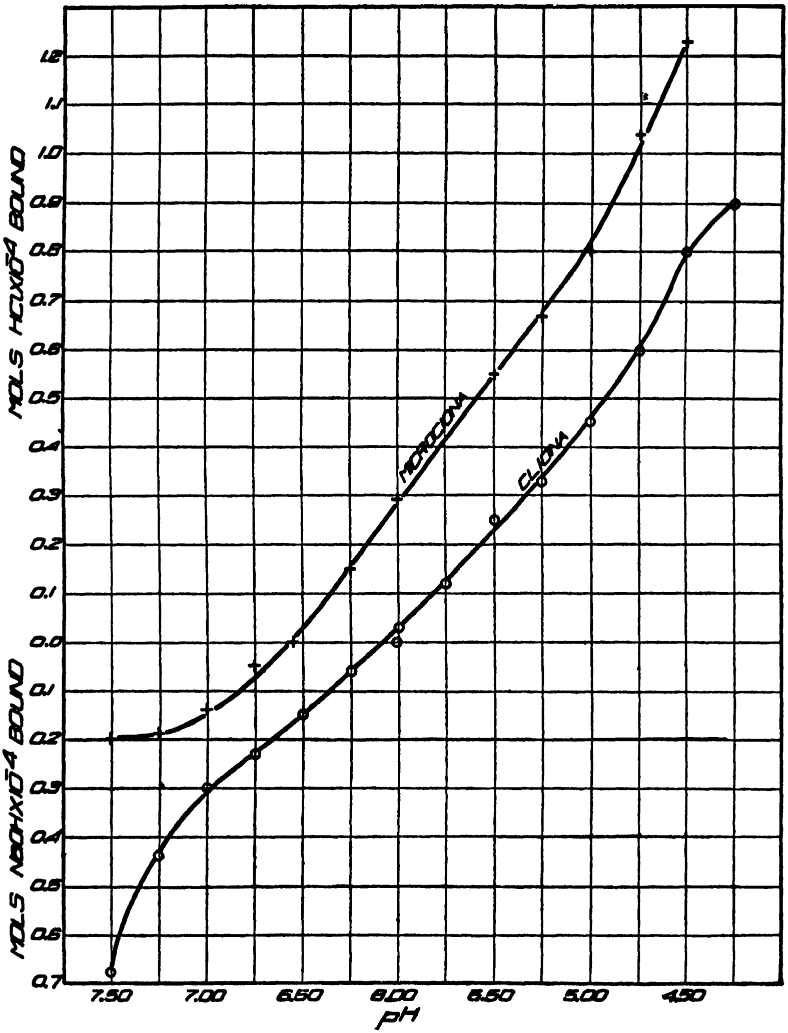

FIG. 3. The acid and base bound by the cells of *Microciona prolifera* and *Cliona celata* at different hydrogen ion activities.

where no base or acid is bound, is of considerable interest for us. It represents the pH of a pure suspension of cells, extracted from the sponge and washed with isotonic NaCl. These pH values for extracted cells are different for *Microciona* and *Cliona*. The cells of *Cliona* are more acidic than those of *Microciona*. We believe that these pH values may be characteristic of the species concerned, provided the comparison is made at a concentration of about 50–60 × 10^8 cells.

The hydrogen ion activity of the original suspension for *Microciona prolifera* is equal to a pH value of 6.55 ± 0.1, and for *Cliona celata* to a pH value of 6.10 ± 0.1.

Passing to the acid range of the acid- or base-binding curve we observe that *Cliona*, being more acidic than *Microciona*, behaves as a weaker base, and binds less acid than the latter. *Microciona*, being more basic, binds more acid for a given change in pH.

The basic part of the acid- or base-binding curve is even more characteristic for the two species of sponges. While *Microciona prolifera* is almost saturated with the small amount of base at a pH value of 7.50, *Cliona* still has a considerable base-binding capacity at that point. It substantiates once more the conclusion reached upon comparison of the two species in the acid portion of the curve; namely, that *Cliona* behaves as a much stronger acid than *Microciona*.

This conclusion, however, is open to one criticism: the suspensions of the cells of *Microciona* and *Cliona*, though containing an equal number of cells, are composed of cells of different sizes. Therefore, the total surface of the cells is different for *Microciona* and *Cliona*.

If the removal of acid or base from the liquid phase, by the cells, is entirely due to the effect of the active surface, the results reached in this investigation would seem to refer to the surface, but not to the chemical properties of the body of the cells.

It is therefore of considerable interest to provide an experimental evidence to prove that the reagents used penetrated inside the cells.

In the course of the investigation upon the cells of *Cliona* it was found that these cells changed their coloration from yellow to dark brown at a pH ranging from 7.3 to 7.4. Upon microscopic examination of the cells, it was observed that the yellow pigmented granules of the cells were responsible for this change in color.

On treating the cells with absolute ethyl alcohol, this pigment can be extracted, and the same change in color reproduced in a test-tube.

These experiments indicate that the cells of *Cliona* in faintly alkaline solution are permeable to our reagents. The reaction is not limited to the surface of the cell.

This evidence cannot, however, be extended to the acid range of titration of *Cliona*, nor to *Microciona* suspensions; but, since we have no reasons for believing that an entirely different physicochemical mechanism is involved in these cases, we are inclined to think that the action of our reagents is not limited to the surfaces of the cells.

We may conclude, therefore, that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Cliona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

IV. CONCLUSIONS.

1. The activity of the hydrogen ion, in a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl per liter, and varied amounts of HCl or NaOH has been investigated. The average value of pK′ for acetic acid in this system is about 4.37.

2. The effect of the addition of various amounts of HCl and NaOH to a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl, and a known number of cells of either *Microciona prolifera* or *Cliona celata* was then studied. It was found that in weak acid solutions *Microciona* behaves as a stronger base than *Cliona*, the former being practically saturated with base at a pH of 7.5. Similar behavior is shown by suspensions of cells to which no acid or base was added: the cells of *Cliona* are more acidic than the cells of *Microciona*.

3. The microscopic examinations of the cells subjected to the treatment with acid or base indicate that the cells of *Microciona* remain alive down to pH 4.50; the cells of *Cliona* sustain greater acidity,—at pH 3.7 they exhibit no signs of cytolysis. Tests for aggregation of these cells showed that this phenomenon is greatly inhibited even by slightly acid solutions.

4. The conclusion is drawn that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Cliona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

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THE GEOTROPIC CONDUCT OF YOUNG RATS.

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I.

If tropistic behavior is to be utilized for ultimate analysis of the inner processes controlling conduct it is quite necessary that the most complete possible mathematical expressions be found for at least several different modes of response. Only in the case of phototropism has any considerable progress in this direction been achieved. We have reference, not so much to the theory of sensory activation, as to the reasonably complete formulation of relationships between the magnitude of the excitatory intensity and the speed and extent of the induced orientation. In this respect the knowledge of geotropism, by contrast, is singularly defective.

For plants, it is found that geotropic excitation is proportional to the sine of the angle of the stimulated part with the horizontal (Fitting, 1905; Pekelharing, 1910). From the relationship between mass of attached leaf and rate of geotropic curvature in horizontal stems of *Bryophyllum*, Loeb (1918, 1924) inferred that the curvature was dependent upon the amount of (gravitationally directed) substance sent into the stem by the leaf. This is obviously consistent with the finding that the "presentation time" for geotropic response is directly proportional to the effective gravitational component (Pekelharing, 1910).

Quite recently the question of geotropic orientation in animals has been reexamined by Cole (1925–26), from the standpoint of the rôle taken by direct action of gravity as leading to tensions produced in muscles which support the organism's weight. With *Helix* Cole was able to show that the speed of upward creeping, after orientation is accomplished, increases with the sine of the angle of inclination of the creeping surface, and thus as the active component of gravity. This leads to the view, substantiated by the effects of forcing such an animal to carry additional loads (Crozier and Federighi, 1924–25; Cole, 1925–26), that orientation is controlled not by some statocyst function but by the differential gravitational pull upon the two sides of the body (Loeb, 1897),—a view earlier advocated for Chitons (Arey and Crozier, 1919), which lack the statocyst of gasteropods.

The information we desired to obtain for the analytical account of geotropism required data upon the amount of upward orientation in a negatively geotropic animal, and the precision of this orientation, as related to the inclination of the creeping surface. For reasons indicated in earlier papers (Crozier and Pincus, 1926–27, *a*, *b;* 1926) we have employed for these experiments young rats of known genetic history, studied during the period of about 2 days which intervenes between the 12th day after birth and the time when the eyelids opened. It happens that with these animals certain new or hitherto ignored features of the geotropic response become apparent and greatly improve the opportunities for investigation. The result seems to indicate quite clearly a direct dependence of orientation upon the distribution of the animal's weight upon the legs of the two sides of the body. Formulæ are derivable describing the orientation with considerable exactness.

We regard it as an interesting fact that, for the first time, a detailed account of a tropism is possible which is based upon experiments with a mammal. This amounts to a sort of reversal of anthropomorphism, and constitutes a decided obstacle for those who would emphasize the greater "simplicity" of lower animals. The simplicity of conduct which permits the mathematical formulation of a mode of behavior is not so much a matter of zoological affinity as it is of dynamical symmetry in the organism and of the choice of experimental conditions which permit the animal to display its potentialities as a machine.

II.

In order to record trails of geotropic orientation each rat was placed upon a fine-meshed wire grid, which permitted a good foothold for creeping. When placed on the creeping plane the axis of the body was at first horizontal, or, occasionally, with the head pointing downward. The tilt of the wire surface was measured on a protractor.

The angle θ was measured when the animal had oriented and was creeping steadily. The correspondence of the wire grid to coordinate paper made it possible to copy the path upon record sheets. The path was indicated either by marking with chalk the position of the rat's axis on the wire, or by placing a straight-edge parallel to the axis. The central stripe due to the hooding factor facilitated such procedure. The path of orientation is a straight line, as shown diagrammatically in Fig. 1, unless, after "hesitation," the rat veers to the opposite side—in which case the angle θ is found to be the same.

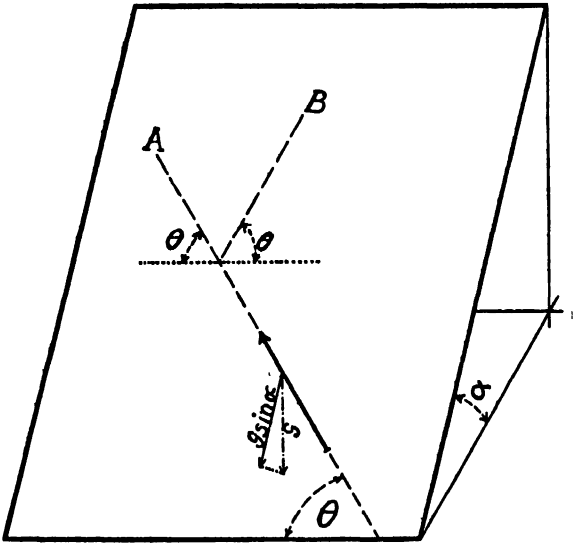

FIG. 1. Diagram showing terms used in description of orientation of rats creeping upon a wire grid inclined at α to the horizontal The position of orientation is defined by the angle θ, the active component of gravity being G sin α. As described in the text, the Path A may be steadily pursued, or the animal may swing to one (B) equally inclined but in the opposite direction.

To obtain data which might be legititimately averaged it is necessary to employ rats as closely comparable as possible. Two albino rats, aged 13 to 14 days, were used in obtaining the records in Fig. 7. Those employed for the other measurements (Fig. 2, etc.) were of different stock, but litter mates. They were brothers of the seventh backcross generation of King inbred albinos with a dark-eyed stock, and were therefore practically homozygous.

III.

The behavior of a rat creeping upon an inclined plane shows one striking peculiarity. It is well known that during the upward locomotion of at least certain negatively geotropic animals the path of progression, especially at inclinations less than 90°, may not be exactly normal to the intersection of the creeping plane with the horizontal. As the inclination is made less, the deviation from the normal increases. This is very obvious in the rats. But there is to be added the further and very important fact that when a rat, at first placed head downward, or with body axis horizontal, orients upward it does so until a certain quite definite angle has been reached, *and then progresses in a straight line.* If creeping becomes interrupted, the rat may show "nervous" random movements of the head. In case these are directed downward, the rat continues creeping along the previous oriented path. But should they be directed upward, locomotion may be pursued at an angle which is exactly the converse of that at first followed. Thus if the angle of orientation was at first 72° to the left, brief creeping may be seen which is more or less irregular but which becomes definite again either at 72° ± to the left, or at 72° ± to the right (*cf.* Fig. 1). This clearly points to the limitation of geotropic orientation by a certain threshold determined through the distribution of the gravitational effect upon the two sides of the body. We shall have occasion to return to this point subsequently.

The results summarized in Table I are derived from twenty tests at each inclination, upon each of two rats from the same litter. Individual quantitative differences undoubtedly exist between diverse genetic strains, but since we are not concerned at the moment with this aspect of the matter we have restricted our account to illustrative material free from this source of confusion. The interpretation of data upon other individuals is entirely consistent with that here detailed. The entries in Table I concern (1) the angle of inclination (α) of the creeping plane to the horizontal; (2) the mean angle of orientation in the creeping plane (θ); and (3) the measure of the variability of θ, employing for this purpose the probable error ($0.8534\ \Sigma v/n\sqrt{n-1}$) expressed as a percentage of the mean.

It is apparent from Table I that the degree of upward orientation

(θ) increases steadily as the inclination of the creeping plane (α) is made greater; and also that the degree of scatter of the individual readings proportionately decreases—that is, the precision of the orientation is enhanced. The minimum inclination leading to a measurable effect lies between $\alpha = 10°$ and $\alpha = 15°$. At 15° the variability of the measurements of θ is disproportionately high, due presumably to the fact that the threshold effect is intrinsically variable from moment to moment. At values of $\alpha > 70°$, orientation is precisely upward ($\theta = 90°$).

TABLE I.

The mean angles of upward orientation (θ) of young rats during creeping upon a surface inclined at angles (α) with the horizontal, and the precision of the respective mean values of θ. The precision is expressed by the probable error as a percentage of the mean (which is equivalent to the coefficient of variation).

$\alpha°$	θ	Variability of θ
		per cent
15	32.6°	8.18
20	44.5°	2.27
25	52.9°	1.87
30	57.4°	1.70
35	64.0°	1.41
40	69.8°	1.18
50	77.9°	1.04
60	84.7°	0.529
70	88.3°	0.351

The extent of orientation (θ) is not directly proportional to the gravitional component in the creeping plane, but to its logarithm. The graph in Fig. 2 shows that the equation

$$\theta = K \log (\sin \alpha) \qquad (1)$$

gives a satisfactory account of the observations; the goodness of fit is probably due to the fact that the individuals used were very closely comparable.

The extent of orientation as a function of α has been measured in certain molluscs by Davenport and Perkins (1897–98) and by Kanda (1916). In the former paper figures are given for the amount of orientation (θ), corrected for random movement, which is visible in *Limax maximus* after 45 seconds exposure upon an inclined plane. Essentially this method was also followed by Kanda (1916), who tabulated the percentage of *Littorina* individuals oriented upward after 1 minute exposure. At best, that is with full correction for movements not directed by geotropism, this procedure can give no quantitative expression for the geotropic excitation; for we should need to have, rather, measurements of the times required to produce a given amount of orientation, expressed either as a constant angle (θ) or as a certain percentage of individuals. For this reason little can be gotten from these data. But it is perhaps significant that the amount of orientation, when expressed in this way, increases more rapidly than log sin α (Fig. 3).

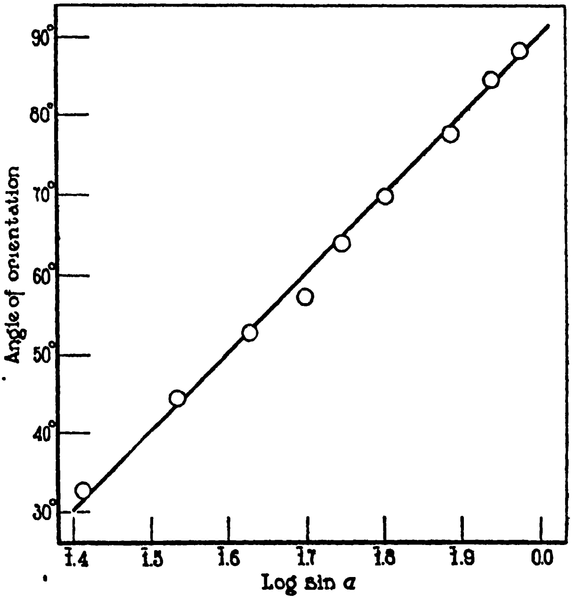

FIG. 2. The angle of upward orientation (θ) is directly proportional to log sin α, where α is the inclination of the creeping surface. With the exception of one point, the mean values of θ do not depart from the line drawn by more than their probable errors. (The points are averages of 40 measurements.)

This could be understood if the speed of orientation, so measured, should depend upon two things, namely speed of creeping and speed of turning, and if each of these separate elements of the act of orientation (Arey and Crozier, 1921; Crozier and Cole, 1923) should be proportional to log sin α. For the data of Davenport and Perkins this is very nearly true for values of α above 15°, and for Kanda's figures below $\alpha = 67° \pm$, but not very much weight can be given to the result.

The *speed* of upward creeping is frequently governed by the intensity of geotropic excitation, and in certain instances can be measured as an index of the effect

CORRECTION.

On page 263, Vol. x, No. 2, November 20, 1926, under *Curve A*, in the legend for Fig. 3, *Ordinate scale at the left* should read *Ordinate scale at the right.* Under *Curve B*, *Ordinate scale at the right* should read *Ordinate scale at the left.*

of gravity. Cole (1925–26) has done this with *Helix*, and concluded that the speed of movement, after orientation is attained, varies as sin α. For the rat, as we shall show presently, the velocity of upward movement decreases as log sin α increases. Question arises as to the existence of any real difference between the two cases. We believe that there is no real difference, because Cole's data show considerable deviation from (K) $(\sin \alpha)$ at low values of α, and especially for the reason that the speed measured was that of the *vertical* ascension. The significance of the latter point lies in the fact that the extent of the average orientation (θ, in the terms previously given), increases with α. Therefore the apparent speed of movement, measured as described, would probably be increased to an illegitimate extent. The data as given show that for *Helix* the "speed of vertical travel" increases faster than log sin α (Fig. 4).

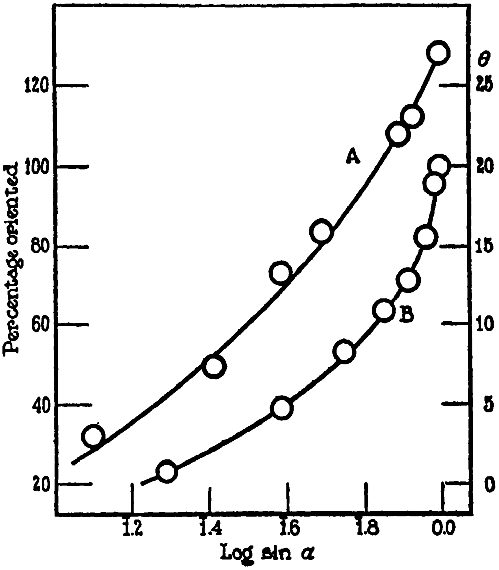

FIG. 3. Curve *A*. Angle of upward gravitational orientation (θ, corrected), for *Limax*, after 45 seconds; (data from Davenport and Perkins, 1897–98). Ordinate scale at the left.

Curve *B*. One series of measurements ("C") of percentages of oriented individuals after 1 minute (*Littorina*); ordinate scale at the right. (Data from Kanda, 1916.)

We have somewhat regretted the form of the relation $\theta = K \log \sin \alpha$, although there are numerous instances of its applicability to other types of response (*cf.* Hecht, 1919–20; and many further cases), partly because of its distressing generality, and partly because of its common association with the Weber-Fechner law. The latter interpretation is frequently misleading (*cf.*, especially, Hecht, 1923-24, 1924-25, and, for the case of phototropism under balanced illumination, Crozier,

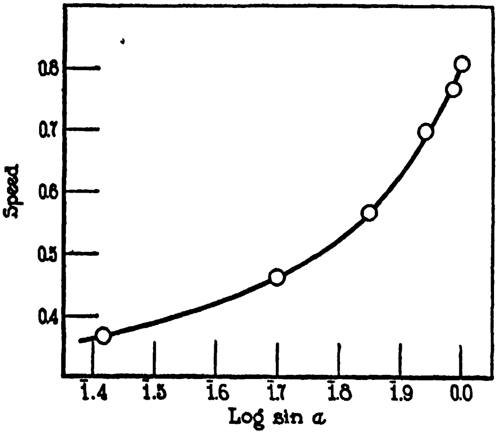

FIG. 4. The speed of ascension of *Helix* as related to the inclination (α) of the creeping surface. (Data from Cole, 1925-26.)

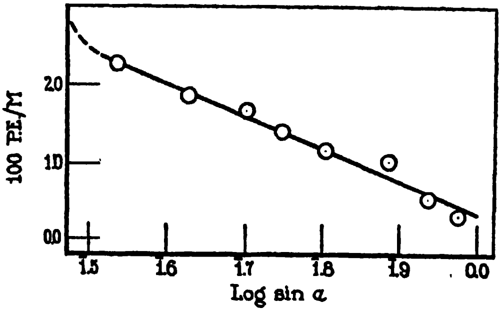

FIG. 5. The decrease in the coefficient of variability (P.E. as per cent of the mean), of the measured values of θ (= angle of upward orientation) is proportional to log sin α.

1926-27). But until more is known of this particular phenomenon we may accept the formula as a convenient empirical expression.

There is additional evidence of its applicability. If our conception of the orientation is correct, then as the gravitational effect is made greater the *precision* of the upwardly directed movement should become correspondingly enhanced. This may be investigated by comparing the numerical expressions for variability of θ as measured at each value of α. If the reduction of variability (V) is proportional to the gravitational stimulus, then

$$- V = K \log \sin \alpha \tag{2}$$

Fig. 5 shows that this relationship is well satisfied, with the exception of the relatively very large variability at $\alpha = 15°$; the deviation here is certainly due to the fact that this inclination is very close to the threshold value for any geotropic effect, as already stated. Thus not only the amplitude or extent, but also the precision of the orientation is determined by the logarithm of the component of gravity acting in the plane of creeping.

IV.

In searching for some clue as to the origin of the logarithmic relation between gravitational stimulus and geotropic response we have noted that if attention be paid to the process of creeping during orientation still another relationship emerges. Until a constant value of θ is attained upon a sloping surface the rat is chiefly *pulled* upward by the forward leg of one side of the body and *pushed* upward by the leg of the opposite side, which is less extended. When θ becomes constant the turning moment vanishes. We may consider, roughly and very crudely, that the orienting power is derived from the actions of levers on the opposite sides, and that the lever arm (x) on the "down" side is shorter than that (y) on the "up" side. Then the torque is responsible for turning upward. When the critical angle of orientation is exceeded, the locomotor action on the two sides of the body becomes equalized, so that if this value of θ is definitely exceeded (*i.e.*, beyond a fluctuating zone, of increasing smallness as α is made larger), the rat is no more constrained than upon a horizontal surface, and is free to turn, should it chance to do so, until an equivalent θ is reached on the other side of the perpendicular. We have already described precisely this behavior. In the line of progression defined by θ, the locomotor effectiveness of the opposed limbs is just barely identical. This means that if we assume the axes of the legs to have mean positions perpendicular to that of the body, then ($x \cos \theta - y \cos \theta$) exactly balances the total downward pull of the animal's weight, where x and y are the "lengths" of the legs as levers on the two sides. Hence,

$$(x - y) \cos \theta = G \sin \alpha,$$

and

$$\frac{\cos \theta}{\sin \alpha} = \frac{G}{(x - y)}.$$

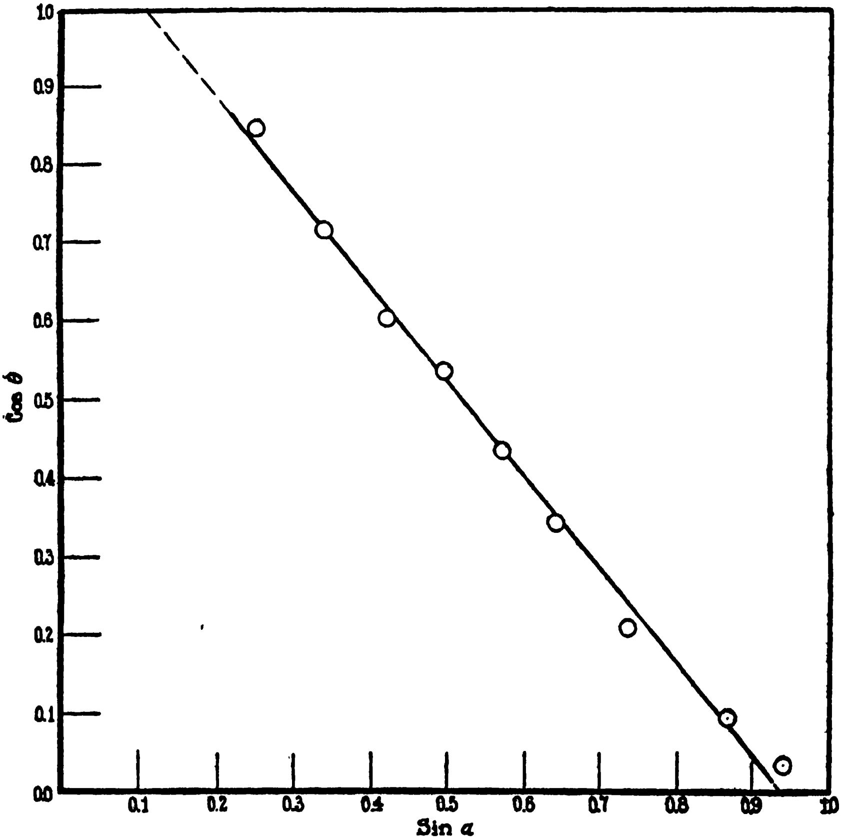

Fig. 6. The cosine of the angle of upward orientation (θ) decreases in direct proportion to the sine of the angle of inclination (α) of the creeping plane.

It was pointed out previously that the legs on the upward side are of course more extended; hence, $y > x$; and if $(x - y)$ is constant for all magnitudes of θ, we have

$$- \cos \theta = K \sin \alpha \tag{3}$$

This relationship is in fact displayed, with unexpected exactness, as shown in Fig. 6, and gives an independent means of checking the significance of the data summarized in Table I. The constancy of the quantity labelled $(x - y)$, derived from the applicability of (3), at once suggests that the difference between the work done by the limbs of the two sides of the body in lifting the animal's weight must be reduced to constant fraction of the total before a stable orientation is attainable. This does not explain, of course, why the animal orients *upward* rather than downward, which may be determined by the inner ears; but it does explain why the amplitude of orientation attains its particular values as the inclination of the surface is varied, and in our opinion it gives an excellent illustration of the muscle-tension theory of orientation.

V.

From Fig. 6, by extrapolation to $\cos \theta = 1$, it is found that the ideal threshold value of α is at about 6.5°; at this point the component of gravity in the plane so slightly tilted is $0.113\ G$. Experimentally, so far as can be determined, the threshold angle is higher than this (10–15°). Hence we may assume, very roughly, because the extrapolation is probably invalid, that when the ratio of the loads on the two sides of the body falls below 10:9, no further orientation occurs. From the derivation of equation (3),

$$(x - y) \cos \theta = W\,G \sin \alpha,$$

$$\frac{\cos \theta}{\sin \alpha} = \frac{W\,G}{(x - y)},$$

it follows that if the weight, $W\,G$, be increased by attaching an additional load with thread to the animal's tail, then, at a given value of α and of θ, the product $(x - y) \cos \theta$ must be larger. The effect of adding such loads is to increase the magnitude of θ; hence it would be expected, from the formula, that $(x - y)$ must increase. The fact is that the locomotion is more labored with added weights attached, and the limbs, especially on the upward side, do become more extended—hence there is good evidence for the occurrence of a change corresponding to an increase in the value of $(x - y)$. The velocity of creeping is decreased in proportion to the added load.

It is of greater interest to see the effect of additional weights upon the extent of the upward orientation (θ). If the effect were a purely mechanical one we would expect to find θ increased in direct proportion to the added weight. But from equation (1),

$$\theta = K \log \sin \alpha,$$

we should expect the change to be such that

$$\theta = K (\log \sin \alpha + \log W \sin \alpha), \tag{4}$$

where W is the added mass, and, when α is constant, θ should increase as log W. Fig. 7 shows that it does. The addition of as little as 1.0 gm. increases the upward orientation somewhat. It is worth while to investigate more fully the relations between θ, α, added load, and rate of locomotion, and in a later paper it is proposed to do so.

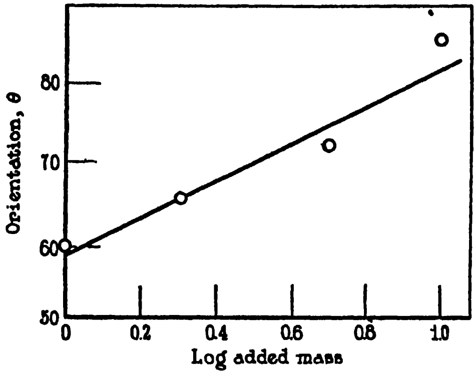

Fig. 7. The angle of upward orientation is proportional to the logarithm of the mass added to the tail of the rat, when the inclination of the creeping plane is constant.

VI.

SUMMARY.

Young rats, old enough to creep well but before the eyelids are open, orient and move upward upon an inclined surface. The angle of geotropic orientation on such a surface (θ) is proportional to the logarithm of the component of gravity parallel to the inclined plane.

This result is compared with the scanty information available for other animals; there is indication that it may be generally valid. The precision of the orientation, measured by the percentage dispersion of the individual measurements, also increases in proportion to the logarithm of this component. The cosine of the angle of orientation decreases very nearly in proportion to the sine of the angle of inclination. A possible interpretation of this is given as involving the idea that upward orientation ceases when the differential pull of the body weight upon the opposed legs reaches a threshold value. Attaching weights (*W*) to the tail causes θ to increase, and in proportion to log *W*.

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THE PENETRATION OF BASIC DYE INTO NITELLA AND VALONIA IN THE PRESENCE OF CERTAIN ACIDS, BUFFER MIXTURES, AND SALTS.

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I.

INTRODUCTION.

It has been shown that the pH value of the cell sap plays[1] an important rôle in the accumulation of the basic dye, brilliant cresyl blue, in the living cell of *Nitella*, and in view of this it is important to study the changes in the rate of penetration produced by varying the pH value of the sap. Experiments of this sort, made by McCutcheon and Lucke,[2] and by the writer,[3] showed that the penetration of ammonia increases the pH value of the sap and decreases the rate of penetration of the dye.

The present paper deals with experiments on the rate of penetration of the dye in presence of acids and buffer mixtures. These experiments are of interest in connection with the hypothesis[1,4] that brilliant cresyl blue exists in aqueous solution in two forms, called for convenience DB and DS. DB, the form which predominates at higher pH values, represents a free base while DS exists predominantly at lower pH values and is a dissociated salt. A normal living cell of *Nitella* is assumed to be chiefly permeable to DB and only very slightly permeable to DS. The present problem is to find the nature of the factors controlling the penetration of DB.

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[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561.

[2] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

[3] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

[4] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 75.

II.

Methods.

Only general methods will be given here: special methods for each set of experiments will be described in connection with the results.

The experiments were carried out in an incubator at 25°C., ± 0.5° having air holes through which diffused light entered.

Only living cells were used. In order to obtain cells in the same condition for experimentation uniformity as to length, thickness, and external appearance was attended to. In the case of *Nitella flexilis* the cells used were taken from the central portion of the plant, midway between the tip and the root. Seasonal changes bring about differences in the permeability of the cells, so that a series of comparative experiments were made on the same lot of cells collected within a short period (near New York in spring unless otherwise stated).

A control experiment was always carried out by removing cells directly from tap water and placing them in the same dye solution as in the case of the test experiment (in which the cells were given some special treatment before being placed in the dye). The rate of penetration obtained from the control experiment was used as a standard of comparison in order to determine the change in the rate of penetration of the dye caused by varying the media in which the cells were placed previous to exposure to the dye solution.

Every determination given represents an average of over 60 experiments and the probable error of the mean is in all cases less than 7 per cent of the mean.

In the case of *Valonia macrophysa* (collected in Bermuda), the procedure was as follows: The clusters of cells were pulled apart and the individual cells were allowed to stand in pans of sea water (which was changed daily) for over 2 weeks in the laboratory (exposed during the day to diffused light). During this period the cell wall at the point of detachment thickened somewhat. This precaution was taken to diminish irregularity in the rate of penetration and the susceptibility of cells to injury upon exposure to solutions.

Small cells (each having a volume of about 0.1 cc.) with one point of detachment, and having practically no attached cells, were chosen. Care was taken to remove adhering organisms or deposits from the surface of the cell.

The detection of an early stage of reversible injury is a very difficult matter, especially with *Valonia*. In the case of *Nitella* an increase in the rate of accumulation of the dye may serve as an indication of a preliminary stage of an injury under certain conditions but this does not seem to be markedly evident in *Valonia*. The exit of halides from the vacuole of *Nitella* or the entrance[5,6] of SO_4 into *Valonia* seems to indicate advanced stages of injury.

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[5] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131.
Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

[6] Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

Cells in good condition are turgid: as they become injured the turgidity diminishes. An experienced experimenter can, to a certain extent, predict the degree of resistance of the cells of *Nitella* and *Valonia* to experimental treatment by the turgidity as ascertained by touching them. This method was used by the writer as a rough guide to the condition of the cells but it does not serve to tell whether injury is reversible. The criterion of irreversibility of injury employed by the writer was as follows: cells which had been exposed to experimental conditions were replaced in the normal medium (*Nitella* in tap water, *Valonia* in sea water), and at intervals during 2 days the rate of mortality was compared with that of the control cells (without exposure to experimental conditions). The criterion of death for *Nitella* was a complete and permanent loss of turgidity, and for *Valonia* either a complete collapse of the cell or disarrangement of chlorophyll and its appearance in the vacuole, so that the greater part of the cell surface appeared colorless. Another test of the condition of the cells is to observe the length of time it takes for them to die in the experimental solutions.

It is not possible, however, to determine experimentally whether the cell was injured at the time of experiment, unless the injury happened to be irreversible. By using these tests an attempt was made to keep the cells uniform during the experiments.

The dye used was made by Grübler and was dissolved in buffer solutions (M/150) in the case of *Nitella* and in sea water in the case of *Valonia*. The pH values of the solutions determined colorimetrically were checked as much as possible by means of the hydrogen electrode. Solutions were not stirred unless otherwise stated.

The determination of the concentration of the dye in the sap was made colorimetrically. With *Nitella*, the cell was gently wiped and was cut at one end, so that the sap could be squeezed out onto a glass slide. With *Valonia* the surface of the cell was punctured with a sharp capillary tube and the sap was drawn up from the vacuole into the tube, from which it was pushed out onto a glass slide. In both cases the sap was drawn up into capillary tubes and the color was matched with capillary tubes of the same diameter containing standard dye solutions.

To determine the pH value of the sap a definite volume was taken by filling a tube for 2 inches with the sap. Indicator solution was drawn up into another tube for a distance of $\frac{1}{20}$ of an inch. The contents of both these tubes were pushed out onto a glass slide and thoroughly mixed. This mixture was then drawn up into a capillary tube and the color matched with that of the capillary tube containing a mixture of standard buffer solution at a known pH value and the same amount of the indicator (the mixture was prepared in the same manner as in the case of the sap). Care was taken to have the least possible contamination of the sap by CO_2 from the breath of the experimenter, as well as to prevent escape of CO_2 into the air, as far as possible.

The color of the indicators changed on standing in a buffer solution containing artificial *Valonia* sap, and also on standing in the natural expressed sap, but the color of the indicators did not change during the time required to determine the pH value of the sap.

The salts in the sap of *Nitella* (about 0.1 M halides) do not seem to affect the indicator seriously, but those in the sap of *Valonia* (about 0 6 M halides) have a very definite effect. In view of the fact that we know so very little about the salt error in general, and possible specific effects of individual salts on these indicators, it will be necessary to study this question carefully before[1] absolute pH values of the sap of *Valonia* can be given.

Another possible source of error in the case of *Valonia* is that the sap is so little buffered that an addition of indicator solution may bring about a change in the pH value of the sap, careful experiments must therefore be made to avoid this error. On the other hand, the sap of the *Nitella* used by the writer is buffered so that this source of error may be negligible. Since only approximate and relative values are desired the pH values of the sap of *Valonia* and *Nitella* given in this paper represent values without a correction for salt error, determined by means of one concentration of indicator dissolved in distilled water of pH 5 8 (approximately the pH value of the sap), or indicator dissolved in alcohol (methyl red). For one series of changes in the pH values only one indicator is used. For example, when experiments were made by exposing cells to a solution of NH_4Cl, brom-cresol purple was used: in the case of cells placed in acid solutions methyl red was used. Brom-cresol green was used to check the values obtained with methyl red, but in view of the fact that the color above pH 5.2 was not satisfactorily matched, only a very rough estimation of the pH value of the normal sap could be made by this indicator. Each indicator is taken from the same stock solution for each series of experiments.

III.

The Decrease in the Rate of Penetration of Dye When the pH Value of the Sap Is Lowered by Entrance of Acetic Acid.

The cells were divided into four lots. One lot was placed in an acetate buffer mixture at pH 5.1, and at the end of 10 minutes the pH value of the sap was compared colorimetrically with that of the normal cell sap. It was found to have decreased[7] from pH 5.5 (normal) to pH 4.9.

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[7] It may be added here that these experiments show that acetic acid enters the vacuole rather easily from an acetate buffer mixture and decreases the pH value of the sap until the internal pH value is less than the external. This agrees with the results obtained by many investigators showing that weak acids enter the living cells. The writer's experiments also show that the pH value of the sap may be raised again when acetic acid is allowed to come out of the vacuole by placing the cells in a solution containing no acetic acid (the more alkaline the external pH value, the more rapid is the rate of exit of acetic acid from the vacuole).

The second lot of cells was placed in the acetate buffer mixture at pH 5.1, and after 10 minutes they were removed, wiped, rinsed for 5 seconds in phosphate buffer mixture at pH 6.6, wiped, and placed in fresh phosphate buffer mixture at pH 6.6. After 1 minute the cells were removed, and the pH of the sap was determined. It was found to be pH 5.2, which is 0.3 pH lower than that of the normal cell sap.

TABLE I.

Comparison of the amount of brilliant cresyl blue in the vacuole when the living cells of *Nitella* are placed for 1 minute in 0 00035 M dye solution at pH 6 6 (M/150 phosphate buffer mixture) after previous exposure to M/150 acetate buffer solution at pH 5 1 for different lengths of time The rate of penetration of dye in the case of cells directly transferred from the tap water to the dye solution is used as the standard of comparison.

External solutions.		In tap water at pH 7 7	In acetate buffer solution 5 sec	In acetate buffer solution 1 min	In acetate buffer solution 10 min
When dye solution is not stirred or changed.	Amount of dye in sap..	M 0 000073	M 0 000069	M 0 000069	M 0 000037
	Percentage decrease on basis of 0 000073 as standard.		5 per cent	5 per cent	50 per cent
When dye solution is stirred and changed every 5 sec.	Amount of dye in sap. .	M 0 00012			M 0 000056
	Percentage decrease on basis of 0 00012 as standard..				47 per cent

The third lot of cells was first exposed to the acetate buffer solution at pH 5.1 for 10 minutes, after which they were removed, wiped, washed for 5 seconds in phosphate buffer mixture at pH 6.6, again wiped, and placed in the 0.00035 M dye solution at pH 6.6 (phosphate buffer mixture). After 1 minute they were removed from the dye solution and the concentration of the dye in the sap was determined colorimetrically, and was found to be 0.000037 M.

The fourth lot of cells was taken directly from the tap water (at pH 7.7) and placed in the same dye solution as the third lot of cells. At the end of 1 minute the concentration of the dye in the sap was found to be 0.000073 M.

Cells thus treated did not live so well as the control cells when replaced in tap water so that in all probability they were more or less injured, but during the experiment the actual appearance of the cells, in respect to chlorophyll arrangement and turgidity, seemed about the same as that of control cells except that the sap appeared slightly murky. Cells kept continuously in the acetate buffer solution began to die in about 3 hours, so that after an exposure of 10 minutes there may have been a very slight injury.

Thus these experiments show that the decrease in the pH value of the sap brought about by acetic acid may be associated with a decrease[8,9] in the rate of penetration of dye amounting to about 50 per cent, as shown in Table I.

This fact is of particular interest in connection with the theory[1,4] that the dye is chiefly in the form of free base (for convenience called DB), at high pH values, and that this alone can penetrate the proto-

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[8] This decrease in the rate of penetration of dye is not due to the lowering of pH value of the external solution immediately surrounding the cell wall as a result of diffusion of acetic acid from the vacuole, because when the experiment is repeated by stirring the external solution, the relative amount of decrease in the rate is about the same as when the external solution is not stirred, as shown in Table I. Furthermore, this decrease is not caused by the adhering of acetic acid to the surface of cell wall in such a manner that it cannot be removed by washing and wiping before the cells are placed in the dye solution, because when the cells are placed in the dye solution, after they have been dipped in the acetate buffer solution only for 5 seconds or for 1 minute instead of 10 minutes, during which exposure the pH value of the sap remains normal, there is no decrease in the rate of penetration of dye, as shown in Table I.

[9] This result is contrary to the result obtained with Cambridge *Nitella* previously described (Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 566, Foot-note 11) where an increase in the rate of penetration took place, but the extent of this increase was not so great (about 25 per cent). Since this work on Cambridge *Nitella* was done in midwinter, the experiments were repeated with the cells obtained in the summer, and it was found that in the majority of cases a decrease took place (about 25 per cent), which is less than the decrease in the case of New York *Nitella*. Such a difference in the behavior of cells may be due to the difference in the condition of the protoplasm.

plasm and enter the vacuole, and that the extent of accumulation of the total dye is dependent on the extent of change of this form, DB, on entering the vacuole into another form, DS, which cannot pass through the protoplasm. In that case we might expect the rate of penetration into the vacuole to be increased when the pH value of the sap is decreased, since with this decrease in the pH value of the sap the ratio of DB/DS decreases in the sap so that as DB enters the vacuole more of it will change to DS, thus causing more DB to enter. But since the experimental results give evidence to the contrary it is evident that the factor which controls the rate of penetration of dye into the vacuole cannot be wholly dependent on the condition of the sap. Under the present experimental conditions the rate of penetration of the dye must be controlled primarily by the effect of the acetate buffer on some other part of the cell. A series of experiments was therefore undertaken to determine the cause of this decrease in the rate of penetration of dye into the vacuole.

IV.

Can the Decrease in the Rate of Penetration of the Dye be Produced without Change in the pH Value of the Sap?

If the theory[4] outlined in Sections I and III were correct we might assume that the decrease in the rate of penetration of dye associated with a decrease in the pH value is due to a change either at the surface or inside the protoplasm caused by the acetate buffer mixture. In that case we might very well expect a decrease in the rate of penetration when the cells are exposed[10] to the solution only long enough for the protoplasmic surface or the interior of the protoplasm to be affected before a change in the pH value of the sap occurs. Unfortunately it is not possible to use the acetate buffer solution for this purpose since the pH value of the sap changes after a very few minutes exposure of the cells to the solution, even at the highest possible pH value (pH 5.4, M/150 acetate buffer mixture). For this reason it was necessary to expose the cells to a phosphate buffer solution at pH 5.4 for 10 minutes, in which the pH value of the sap remained unchanged, and to compare the rate of penetration of dye in the case of cells thus treated with the rate in the case of cells previously exposed for the same length of time in an acetate buffer solution at pH 5.4 where the pH value of the sap decreased from 5.5 (normal) to 5.0. As shown in Table II, there is about the same amount of decrease[11] in the rate of penetration whether the pH value of the sap is lowered or remains normal. The mortality of the cells thus treated is lower than that of the cells exposed to the acetate buffer mixture.

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[10] The detailed description of the method of experimentation will be omitted hereafter since it is given in Section III. It may be repeated here that in all cases the cells were washed for about 5 seconds in a buffer solution at the same pH value as that of the dye solution before they were placed in the dye solution and the cells were invariably wiped before they were placed in any solution. Cells were exposed for 1 minute in the solution of dye, 0.00035 M made up with phosphate buffer mixture at pH 6.6 unless otherwise stated.

TABLE II.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when the cells are placed in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after a previous exposure of the cells for 10 minutes either to the M/150 acetate buffer solution (when the pH of the sap is decreased) or to the M/150 phosphate buffer solution (when the pH value of the sap is not decreased).

External solutions.	In tap water at pH 7.7.	In acetate buffer solution at pH 5.4.	In phosphate buffer solution at pH 5.4.
Amount of dye in sap	M 0.000073	M 0.000039	M 0.000041
Percentage decrease on basis of 0.000073 as standard		47 per cent	44 per cent

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[11] Since there is about the same amount of decrease in the rate of penetration of dye whether the pH value of the sap is decreased or not, such a decrease cannot be due primarily to the decreasing of the pH value of the film[4] of liquid between the protoplasmic surface and the cell wall as result of diffusion of acetic acid from the vacuole into the film. This film is the only part of the external system which affects penetration since it alone determines the number of dye molecules striking the protoplasmic surface. The condition of the external solution may be regarded as of importance only in so far as it affects this film.

V.

Is the Decrease in the Rate of Penetration of the Dye Due to the Effect of H Ions on the Surface or to Their Penetration (as Ions) or to the Entrance of Acids in Undissociated Form?

The decrease in the rate just described was about the same whether the pH value of the sap was lowered or not, and this suggests that the decrease in the rate might be due to the direct action of H ions on the surface or their penetration as ions when the pH value of the external solution changed from pH 7.7 (tap water) to pH 5.4 (buffer solutions). If this assumption were correct we might expect the rate of penetration to be about the same whether the cells were previously exposed to tap water, to phosphate, or to borate buffer solutions at pH 7.7 providing equal numbers of hydrogen or hydroxyl ions enter in each case.

In order to test this the rates of penetration of dye were compared among the three groups of cells previously placed[10] for 10 minutes (1) in tap water (control), (2) in phosphate buffer solution, and (3) in borate buffer solution, all at pH 7.7, and it was found (as shown in Table III) that with phosphate buffer solution there was about 30 per cent less dye in the vacuole than in the case of the control, and with borate buffer about 13 per cent less dye (which may not be significant since the probable error of the mean is rather high).

The experiments were extended to higher pH values, pH 8.1 and 7.3, and it was found, as shown in Table III, that the rate of penetration of dye is again lower in the case of cells previously exposed[10] to the phosphate buffer solution than that in the case of cells exposed to the borate solution. Such a difference in behavior between the borate and the phosphate buffer mixtures cannot be due to the effect of H or OH ions as such on the cell, since the pH value is the same in both these solutions.

Other experiments are therefore needed to determine just what causes this difference.

This difference between the phosphates and the borates, as affecting the rate of penetration of dye, is not due to the difference in the effect[12] of these buffer mixtures directly on the dye, as is proved by the fact that when the cells are transferred[10] directly from the tap water to the dye solution at pH 7.7 made up (1) with borate buffer mixture and (2) with phosphate buffer mixture, the rate of penetration of the dye is the same, as shown in Table IV.

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[12] It is not possible, unfortunately, to determine if there is an effect of acetate buffer mixture on the dye, since it is impossible to determine the penetration of dye at a pH value lower than pH 6.2 in the case of *Nitella*.

TABLE III.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when cells were previously exposed to M/150 borate and phosphate buffer solutions at different pH values for 10 minutes after which they were placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture).

External solutions.	In tap water at pH 7.7.	In phosphate buffer solution at pH 7.7.	In borate buffer solution at pH 7.7.	In phosphate buffer solution at pH 8.1.	In borate buffer solution at pH 8.1.
	M	M	M	M	M
Amount of dye in sap.................	0.000079	0.000055	0.000069	0.000059	0.000079
Percentage decrease on basis of 0.000079 as standard........................		30 per cent	13 per cent	26 per cent	0 per cent

External solutions.	In phosphate buffer solution at pH 7.3.	In borate buffer solution at pH 7.3.	In phosphate buffer solution at pH 6.6.	In borate buffer solution at pH 8.7.
	M	M	M	M
Amount of dye in sap.................	0.000052	0.000069	0.000048	0.000079
Percentage decrease on basis of 0.000079 as standard........................	35 per cent	13 per cent	39 per cent	0 per cent

TABLE IV.

Comparison of the amount of brilliant cresyl blue in the sap after 1 minute in 0.00017 M dye solutions at pH 7.7 made up with different buffer mixtures (M/150).

External dye solutions.	Borate buffer mixture.	Phosphate (ordinary) $Na_2HPO_4 + KH_2PO_4$.	Phosphate (lacking K) $Na_2HPO_4 + NaH_2PO_4$.
	M	M	M
Amount of dye in sap..........	0.00041	0.00035	0.00040

This difference furthermore is not due to a specific action of the K in the phosphate buffer mixture since the experiments were repeated[10] with the solution made up with Na_2HPO_4 containing NaH_2PO_4 instead of KH_2PO_4, at pH 5.4 and 7.7 and the same result was obtained, as shown in Table V.

TABLE V.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella* after 1 minute exposure to 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) following a 10 minute exposure to the M/150 phosphate buffer mixtures consisting of Na_2HPO_4 and either KH_2PO_4 or NaH_2PO_4.

External solutions.	In tap water at pH 7.7.	In Na_2HPO_4 + KH_2PO_4 at pH 7.7.	In Na_2HPO_4 + NaH_2PO_4 at pH 7.7.	In Na_2HPO_4 + KH_2PO_4 at pH 5.4.	In Na_2HPO_4 + NaH_2PO_4 at pH 5.4.
	M	M	M	M	M
Amount of dye in sap.................	0.000084	0.000055	0.000059	0.000048	0.000048
Percentage decrease with 0.000084 as standard..........................		33 per cent	29 per cent	42 per cent	42 per cent

TABLE VI.

Comparison of amoumt of brilliant cresyl blue in the vacuole when cells of *Nitella* (autumn) are placed in 0.00004 M dye solution (stirred) at pH 7.7 (M/150 borate buffer) for ½ minute, after they have been exposed for 10 minutes to various solutions.

External solutions.	Tap water pH 7.7.	Boric acid pH 4.8.	Hydrochloric acid pH 4.8.	Phosphoric acid pH 4.8.	Phosphate buffer pH 5.4.
	M	M	M	M	M
Dye in sap..........................	0.000072	0.000076	0.000069	0.000058	0.000042
Percentage decrease on basis of 0.000072 as standard.......................		5 per cent increase (?)	5 per cent	20 per cent	42 per cent

The inhibiting effect of phosphate buffer mixtures is greater the lower the pH value, as shown in Table III. It may be that this is due to the greater amount of phosphoric acid present in the buffer mixture, if we assume that as a weak acid it penetrates the protoplasm as undissociated molecules and dissociates after entering and lowers the pH value of the protoplasm, so that when cells are subsequently placed in a dye solution there will be less DB (since DB changes to DS more at a low pH value) in the protoplasm than in the case of the control cells which are transferred directly from tap water to the dye solution. The rate of penetration of DB from the protoplasm to the vacuole will therefore be less than in the case of the control cells.

This assumption[13] is partly supported by the following result. When the cells[13] are exposed for 10 minutes to three separate solutions, (1) hydrochloric acid, (2) boric acid, (3) phosphoric acid, all at pH 4.8, and then placed in the dye solution[13] (borate) for $\frac{1}{2}$ minute, the rate of penetration of dye (as compared with that of the control cells, which are transferred directly from tap water to the same dye solution) in the case of hydrochloric acid and boric acid is about the same as that of the control. This indicates that H ions do not affect[13] the cell and that if boric acid enters the cell as undissociated molecules it does not afterward dissociate sufficiently to lower the pH value to any appreciable degree. Phosphoric acid behaves differently in that the rate of penetration of the dye in the case of the cells exposed previously to this acid is found to be about 20 per cent lower than that of the control, which indicates that phosphoric acid enters the protoplasm and then dissociates sufficiently to lower the pH value or else that it has a specific effect on the surface (Table VI).

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[13] There are several other possible explanations, for example:

(1) On the basis that phosphoric acid enters more rapidly than boric. We are unable to prove this experimentally, for which reason the explanation described in the text is used instead.

(2) On the basis that a weak acid enters the protoplasm as undissociated molecule and by dissociating lowers the pH value of the protoplasm and that when such cells are removed from the buffer solution to the dye solution, the weak acid diffuses out from the protoplasm into the film of liquid between the protoplasmic surface and the cell wall, and lowers the pH value of the film thereby decreasing the ratio of DB/DS in the film. This will explain the difference between boric acid and phosphoric acid, in that boric acid does not change the pH value of the film since it is too weak an acid, while phosphoric acid is sufficiently strong to bring about this change. But this assumption is not so satisfactory as the one described in the text when we consider the fact that there is an inhibiting effect on the rate of penetration of dye even with cells previously exposed to a phosphate buffer solution at pH 8.1 and then placed in dye solution at much lower pH value (pH 6.6). In such a dye solution one would expect further entrance of phosphoric acid into the cell, rather than exit of the acid from the protoplasm to the exterior of the cell. These cells were collected in autumn.

The dye solution was stirred. At a lower pH value both phosphoric acid and hydrochloric acid have an inhibiting effect which is greater in the case of the former.

The cause of the decrease brought about by the phosphate buffer mixture may be threefold, (1) due to undissociated phosphoric acid, (2) due to the Na and K salts present in the buffer mixture, and (3) due possibly to certain anions.

It may be of interest to add here the following. When cells (collected in Cambridge) are exposed for 10 minutes to solutions at different concentrations (0.05 M to 0.006 M) of NaCl, LiCl, KCl, Na_2SO_4, and $NaNO_3$ made up in distilled water, after which they are washed in distilled water for 5 seconds, wiped, and are placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) for 1 minute, the rate of penetration is considerably decreased as compared with the control. If cells are placed directly for 1 minute (without such treatment) in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) containing any one of these salts, the rate is found to be slightly higher than in the case of cells placed in dye solution containing no salt.

Solutions of $MgCl_2$, $MgSO_4$, $CaCl_2$, $LaCl_3$, and $LaNO_3$ all behave alike, in that when cells are exposed to these solutions for 10 minutes and then transferred to the dye solution, the rate of penetration of dye is about the same as the control. When cells are placed without such treatment in dye solutions containing any one of these salts ($LaCl_3$ omitted), the rate is found to be somewhat higher than that of the control.

Thus there is evidence for the inhibiting effect[14] of the salts with monovalent base cations which is not easily reversible, since the effect may be brought about by placing the cells in 0.01 M NaCl solution for 5 minutes, but this effect does not disappear after the cells have been transferred to distilled water and left for over an hour. This effect, however, may be readily removed if cells are placed in a solution of salt with bivalent or trivalent cations, such as $MgCl_2$ and $LaCl_3$ at certain concentrations.

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[14] The experiments described in the text (see Table III) show that the borate buffer mixtures have no inhibiting effect on the rate of penetration of dye. In view of the fact that the borate buffer mixtures at higher pH values contain a considerable amount of Na, there is an apparent discrepancy between the results obtained in this case and those in the case of NaCl solutions in which there is a considerable inhibiting effect due to the presence of Na (this discrepancy was mentioned in the writer's previous paper (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, No. 1)). This, however, may be due to the fact that in the case of cells previously exposed to the borate buffer mixtures the dye was made up with phosphate buffer mixtures which seem to diminish the inhibiting effect of Na, while in the case of cells previously exposed to NaCl solutions the dye used was made up with borate buffer mixture which does not seem to have this effect. The following experiments may make this clear. When cells previously exposed to (1) 0.01 M NaCl and (2) to 0.005 M sodium borate solutions for 10 minutes were washed for 5 seconds in distilled water, wiped, and placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture), there is a considerable inhibiting effect

Further experiments are being carried out on this subject by the writer.

VI.

Experiments on Valonia.

The experiments have been repeated with *Valonia macrophysa* but the results described below are approximate and show only relative values, owing to the fact that the pH value of the sap cannot be accurately determined without special experiments and that the sea water not only shifts the dissociation constant considerably, but seems to change the nature of the dye, especially at lower pH values.

A. The Effect of Aqueous Ammonia (Free and Combined).—Since the method of determining the change in the pH value after placing cells in solutions has been described in detail in Section II, it will be omitted here. When cells of *Valonia* were placed for 1 hour in sea water containing 0.003 M NH_4Cl solution, the pH of the sap increased from 6 (normal) to 6.6 (determined colorimetrically by using bromcresol purple). When such cells were replaced in sea water and left for 1 hour the pH value decreased from 6.6 to 6.1.

One group of cells was placed in sea water containing 0.00035 M dye, a second group in sea water containing 0.003 M NH_4Cl and

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which is slightly greater with (1) than with (2). If such cells (1) and (2) are placed in 0.00014 M dye solution at pH 7.7 (phosphate buffer solution) they show no inhibiting effect at all.

At a higher concentration of NaCl (0.05 M) this inhibiting effect is not removed in 0.00014 M dye made up with phosphate buffer mixture at pH 7.7.

The inhibiting effect of previous treatment with the phosphate buffer mixture at pH 5.4 (Table II) is increased in 0.00014 M dye solution at pH 7.7 made up with borate buffer mixture.

0.00035 M dye. A third group of cells was first exposed for 1 hour to sea water containing 0.003 M NH_4Cl and then transferred to the dye solution used in the case of Group 1. After 1 hour there was a decided decrease in the rate of penetration of dye in the case of cells placed in the dye solution containing NH_4Cl (Group 2) and also in the case of cells previously exposed to NH_4Cl solution (Group 3), as compared with the control (Group 1). These results show that the presence of ammonia in the cell brings about a decrease in the rate of penetration of dye. Whether this decrease is entirely due to the increase in the pH value of the sap in the presence of ammonia or due partly to the former and partly to the presence of ammonia in the protoplasm (at the surface or the interior), it is not possible to determine. These results confirm those obtained with *Nitella*[3] (see Section I).

B. Effect of Acetic Acid and HCl at pH 5.9.—Let us first see if the same results may be obtained as with *Nitella* when the pH value of the sap is decreased by entrance of acetic acid. When cells were placed in sea water containing acetic acid at pH 5.9, the pH value of the sap decreased in 1 hour from 5.5 (normal) to 4.8 (methyl[15] red used as an indicator). The pH value of the sap thus decreased was found to be raised to the normal when such cells were placed in sea water for 20 minutes. When cells whose pH value had been thus decreased were placed for 20 minutes in sea water containing 0.00035 M dye, the amount of dye in the sap was less[16] than in the sap of cells transferred directly from the sea water to the same dye solution (control). These experiments show that there is a decrease in the rate of penetration of dye when the pH value of the sap is decreased by entrance of acetic acid, which agrees with the results discussed in Section III on *Nitella*.

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[15] Difference between the determination of the pH value of the sap made with brom-cresol purple and with methyl red lies in the fact that the effect of salt on the indicator is not corrected. The explanation of the use of the indicators is described in Section I.

[16] Brooks exposed cells of *Valonia macrophysa* to sea water (1) containing NH_4Cl until the pH value of the sap increased, and (2) containing CO_2 until the pH value of the sap decreased, after which they were placed in sea water containing 2, 6, dibromophenol indophenol, and found that the rate of penetration of dye decreased with (1) and increased with (2). She interprets these results on the basis that the rate of penetration of dye is affected by the change in the pH value of the external solution surrounding the cell as a result of diffusion of (1) NH_4Cl and (2) CO_2 from the vacuoles. (See Foot-note 6.)

Let us now see if a decrease can be brought about without a change in the pH value of the sap. Cells were exposed for 1 hour to sea water containing HCl at pH 5.9, after which they were placed in 0.00035 M dye for 20 minutes. The rate of penetration in this case was found to be less than the control but the extent of decrease in the rate is not so great as it was in the case of cells exposed to acetic acid.

When cells are placed for 1 hour in sea water containing 0.0007 M dye at pH 5.9, (1) containing acetic acid and (2) containing HCl, the rate of penetration was found to be higher with acetic acid than with HCl.

C. Effect of Sea Water at pH 6.5 Containing either Acetic Acid or HCl (No Change in the pH Value of the Sap).—The question now arises as to what will happen if we put cells in sea water containing acetic acid at a pH value at which there is no decrease in the pH value of the sap. Cells were placed in sea water at pH 6.5 containing acetic acid for 1 hour after which they were transferred to sea water containing 0.00035 M dye for 20 minutes: the pH value of the sap remained normal. When the rate of penetration of dye in the case of the cells thus treated was compared with that of the control (cells directly removed from the sea water and placed in the same dye solution), it was found to be the same. In the case of the cells previously exposed to sea water containing HCl at pH 6.5 the rate of penetration of dye was also found to be the same.

Cells placed in 0.00017 M dye in sea water at pH 6.5 containing (1) acetic acid and (2) HCl, showed no difference in the rates.

Thus these experiments show that in the case of *Valonia* also the rate of penetration of dye may be retarded when (1) the pH value of the sap is decreased in presence of acetic acid, and (2) the pH value of the sap is increased in presence of NH_3, when cells are exposed to these solutions before they are placed in the dye solutions.

SUMMARY.

When living cells of *Nitella* are exposed to an acetate buffer solution until the pH value of the sap is decreased and subsequently placed in a solution of brilliant cresyl blue, the rate of penetration of dye into the vacuole is found to decrease in the majority of cases,

and increase in other cases, as compared with the control cells which are transferred to the dye solution directly from tap water. This decrease in the rate is not due to the lowering of the pH value of the solution just outside the cell wall, as a result of diffusion of acetic acid from the cell when cells are removed from the buffer solution and placed in the dye solution, because the relative amount of decrease (as compared with the control) is the same whether the external solution is stirred or not.

Such a decrease in the rate may be brought about without a change in the pH value of the sap if the cells are placed in the dye solution after exposure to a phosphate buffer solution in which the pH value of the sap remains normal. The rate of penetration of dye is then found to decrease. The extent of this decrease is the greater the lower the pH value of the solution.

It is found that hydrochloric acid and boric acid have no effect while phosphoric acid has an inhibiting effect at pH 4.8 on stirring.

Experiments with neutral salt solutions indicate that a direct effect on the cell (decreasing penetration) is due to monovalent base cations, while there is no such effect directly on the dye.

It is assumed that the effect of the phosphate and acetate buffer solutions on the cell, decreasing the rate of penetration, is due (1) to the penetration of these acids into the protoplasm as undissociated molecules, which dissociate upon entrance and lower the pH value of the protoplasm or to their action on the surface of the protoplasm, (2) to the effect of base cations on the protoplasm (either at the surface or in the interior), and (3) possibly to the effect of certain anions. In this case the action of the buffer solution is not due to its hydrogen ions.

In the case of living cells of *Valonia* under the same experimental conditions as *Nitella* it is found that the rate of penetration of dye decreases when the pH value of the sap increases in presence of NH_3, and also when the pH value of the sap is decreased in the presence of acetic acid. Such a decrease may be brought about even when the cells are previously exposed to sea water containing HCl, in which the pH value of the sap remains normal.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.

THE RÔLE OF CERTAIN METALLIC IONS AS OXIDATION CATALYSTS.

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It has long been known that metals in some way are very closely connected with the processes of respiration and oxidation in both plants and animals, and the mode of their action has been the subject of a great deal of research and discussion. The most important of these metals is undoubtedly iron, and the recent work of Warburg (1925) and Meyerhof (1924) has gone far toward elucidating its mode of action.[1] Iron as an oxygen carrier is present in the hemoglobin of the blood of mammals and has been the subject of much investigation. It is not within the province of this paper, however, to discuss this aspect of the matter, treatment of which may be found in text-books of general physiology.

Next to iron, in occurrence and in importance, is copper, although the latter has not received the careful and attentive research which has been bestowed on the former.[2]

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[1] The book by Meyerhof (Chemical dynamics of life phenomena, 1924) contains an excellent summary of investigations up to a very recent date. Reference may also be made to the individual papers of these authors, and particularly, in English, of Warburg (1925). Crozier (1924–25) has analyzed the data of various investigators who have worked with oxidation systems, from the point of view of the effect of temperature. He finds that many oxidation reactions, including several which are undoubtedly catalyzed by iron, have a critical thermal increment close to 16,000. Hecht (1925–26) explains on this basis the latent period of the photic response in *Ciona*, the critical thermal increment of which is nearly 16,000, as a reaction catalyzed by iron and probably an oxidation. The fact that many oxidation systems and many reactions catalyzed by iron have the same increment may be taken as further evidence that iron is intimately connected with oxidation catalysis.

[2] The toxic effects of copper have been widely observed and discussed (see papers by Cook, 1925–26). In fact, the striking and obvious toxic action of copper has more or less obscured the possibility that this element may be of fundamental importance in the catalysis of normal oxidation reactions.

That copper occurs normally in a large number of organisms has been shown by Maquenne and Demoussy (1920) for plants and Muttkowski (1920–21) and Rose and Bodansky (1920) for animals. In animals it is usually found in the hemocyanin, or oxygen-carrying fluid, of arthropods and crustaceans. Hemocyanin is considered analogous to hemoglobin, with copper taking the place of the iron. Henze (1904–05), working on octopus blood, decided that oxidations were catalyzed by the copper. Alsberg and Clark (1914), using *Limulus*, concluded that "With the aid of copper, oxygen may, perhaps, be transferred catalytically within the organism." Glaser (1923) found considerable copper in *Arbacia* eggs; he thinks its function is partially to inactivate certain enzymes.

Of the other metallic elements manganese (Bertrand, 1897, and later papers) is the only one which has been mentioned as a substitute for iron and copper. McHargue (1926) states that manganese is found very frequently in chlorophyl-bearing tissues and assigns to this metal an important rôle in photosynthesis. In molluscan bloods Mn may appear to take the place of Cu. However, its function, if not its occurrence, is rather problematical, and it does not take rank in prominence with the other two. Some reasons will be advanced, based on the present work, for believing that iron and copper, if not the only two elements possible, are nevertheless the two elements which are, chemically, especially well adapted to the rôle of oxidation catalysts.

One method of approach to the problem of biological oxidations is the measurement of respiration in organisms. Another is the attempt to duplicate, as far as possible, the conditions existing in the cell by means of inorganic chemical systems where the conditions may be controlled. The first method has led to the conclusion that iron, and to a lesser extent copper, is the catalytically active substance. The second method is more apt to furnish data regarding the mechanism of the reactions involved and is the one used in the present investigation.

II.

In order to duplicate the essential conditions in living systems it is necessary to have an oxygen-rich substance (a peroxide), a catalyst, and an easily oxidizable substance. Ray (1923–24) has proceeded according to this principle and has investigated the system iron-hydrogen peroxide-unsaturated fatty acids from the point of view of the effect of anesthetics. It was thought best here to use a combination which reacted quite rapidly, and therefore pyrogallol was used as the oxidizable substance. One of the end-products of the reaction is carbon dioxide, the rate of production and amount of which may be accurately measured.

The experimental measurements were made with an Osterhout respiration machine the principles of which have been described in an earlier paper (Cook, 1925–26). Briefly, there is a closed system of tubes through which air is forced by a pump, from a reaction chamber to a tube containing an indicator (phenolsulfonphthalein) and thence by another route to the reaction chamber again. The indicator is decolorized by the carbon dioxide and the color is restored by switching the current of air through a U-tube containing sodium hydroxide.

In carrying out the present series of experiments a definite amount of pyrogallol was dissolved in water in a large test-tube (the reaction chamber); the tube was placed in its proper position in the circuit of the machine, and the carbon dioxide present in the solution was cleared out. Then through a separatory funnel a mixture of the metal salt and hydrogen peroxide was run into the reaction chamber. These two constituents were mixed *immediately* before being run in, so that there might be as little reaction between them as possible before striking the pyrogallol solution; the error here involved is entirely negligible because the reaction between the metal and the peroxide is relatively slow. Then the machine was started and the carbon dioxide produced was measured practically from the start of the reaction.

In order that there might be no possibility of the walls of the container exercising a catalytic effect, or otherwise disturbing the reaction, the inside of the reaction chamber was coated with paraffin and the coating renewed frequently. Furthermore, the indicator solution was replaced after every experiment in order to guard against any contamination by volatile organic acids which might be produced during the oxidation of the pyrogallol. Finally the reaction chamber was placed in a water bath and the temperature was kept uniformly at 25°C. in all the experiments here reported.

Unless the concentration of the reactants was purposely varied the mixtures were made up by dissolving 0.1 gm. of pyrogallol in 44 cc. of water (distilled) to which was added 1 cc. of hydrogen peroxide and 10 cc. of the metal salt in the desired concentration. The hydrogen peroxide was all taken from the same bottle and retained its strength at approximate constancy throughout the entire series of experiments.

Since the hydrogen ion concentration of the medium affects the rate of oxidation of pyrogallol it was ascertained colorimetrically that a mixture of 0.1 gm. of pyrogallol and 1 cc. of the peroxide in 44 cc. of water has a pH of approximately 5. Since most of the metal salts here used have an acid reaction there is no doubt that all the present experiments were performed in an acid medium. The pyrogallol will not absorb oxygen as rapidly in an acid as in an alkaline medium, but since the hydrogen ion concentration did not vary to any great extent, and always remained on the acid side of neutrality, the relative values obtained are not invalidated. It would of course be impossible to conduct experiments by the indicator method with an alkaline medium in the reaction chamber.

It is customary when using the indicator method with an organism to express the results as a rate curve based on the normal rate of respiration as 100 per cent. Such a procedure is impossible here since there is no "normal rate." In fact there is no production of carbon dioxide previous to the start of the reaction.[3] It is necessary, therefore, to find some other method of expressing the results. This may be done by using the absolute amounts of carbon dioxide produced. Ray (1923–24) has developed this method with the assistance of E. J. Cohn and has calculated the actual quantity of the gas. Here it is not essential to know the actual quantities in mg. Arbitrary units are satisfactory since all the results are relative and may be compared with each other even if we do not know the exact amounts. If the same amount of sodium bicarbonate is always present in the indicator solution and the buffer standards always remain constant, then it will always take the same amount of carbon dioxide to decolorize the indicator. We may then take this amount as a unit and calculate the number of units produced in a given time, or the length of time to produce 1 unit. But we must remember that the reaction is also proceeding during the time that the color of the indicator is being restored and include this time. For example, if it takes 30 seconds to decolorize and 30 seconds to restore the color then 2 units of carbon dioxide are formed per minute. Or if in 10 minutes there are four periods of decolorization 2 minutes long and four periods of 30 seconds to restore the color, then 5 units will have been formed during the 10 minutes.

Using this method the total amount of carbon dioxide produced can be plotted against the time and an integral curve obtained. If the equation of this curve is known then the rate curve can be derived therefrom.

III.

Since this investigation has been primarily on the effect of copper, this element will be treated in a separate section. Fig. 1 shows typical curves obtained with 1 cc. of hydrogen peroxide, 0.1 gm. of pyrogallol, and various concentrations of copper chloride, in 44 cc. of water. The curves represent the total amounts of carbon dioxide produced, plotted against time. The slope of such a curve at any point indicates the rate of production of carbon dioxide. It will be seen that with very dilute copper the rate is at first relatively rapid, but soon falls off until it approaches zero. With concentrated solutions the rate decreases continually, but since the earlier portion of the curves is most important it was not considered necessary to follow the course of the reaction entirely to completion. In fact to do so with concentrated copper solutions would require many hours if not days of continuous observation. The upper curves, therefore, are incomplete.

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[3] Pyrogallol is oxidized by hydrogen peroxide and metal salts separately at such a slow rate (if at all) that it cannot be detected by this method. Hence we may say for practical purposes that there is no production of carbon dioxide unless all three constituents are present.

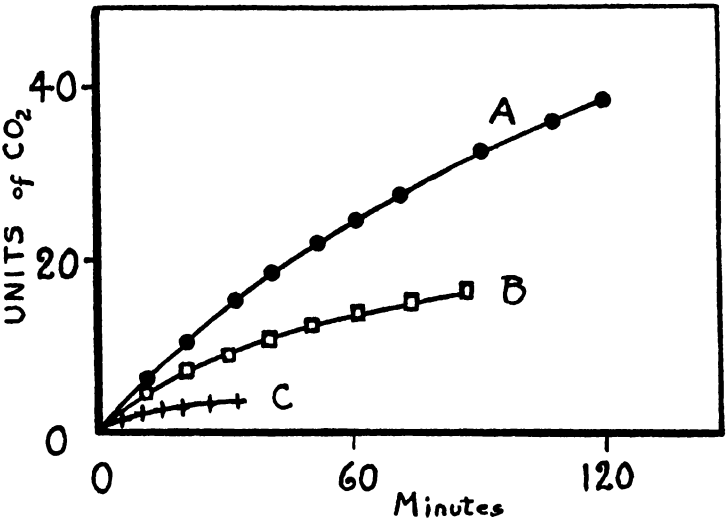

FIG. 1. The quantity of CO_2 produced by 0.1 gm. of pyrogallol, 1 cc. of H_2O_2, 44 cc. of water, and 10 cc. of

in Curve *A*, 0.0003 M; $CuCl_2$
" *B*, 0.0001 M; "
" *C*, 0.00001 M; "

The ordinates represent arbitrary units of CO_2 and the abscissæ minutes. Each curve is the average of three or more experiments.

In order to compare and analyze curves of this sort the equations should be known. The attempt was made to fit the curves with the equations of a monomolecular and bimolecular chemical reaction. Although in some instances an approximate constant can be obtained, the correspondence is not very close, and these equations have very little significance in the present connection. However, the curves can be fairly closely fitted by the general equation for the hyperbola $y = x/(a + bx)$. In all the experimental cases, when x/y is plotted against x the resulting figure is a straight line. There are individual deviations of slight extent but these are irregular in their occurrence and indicate that the experimental curves are fitted only approximately by the general equation $y = x/(a + bx)$. Even though the correspondence is only approximate, however, it is sufficiently close to permit the use of the equation in a purely empirical way for the comparison of the curves. In considering the present data the special equation may be used $a = t/(p + bt)$, where a is the amount of carbon dioxide produced after time, t, and p and b are constants.

The constants p and b must be determined for each different curve and when determined[4] will be an index to the characteristics of the curve, within the same limits as were suggested with respect to the accuracy of the general equation as applied to these cases. The constants, with these reservations, may be used to compare the action of different concentrations of the reagents. To evaluate the constants the method of least squares was used.[5] In the experimental curves we are particularly interested in the total amount of carbon dioxide which will be formed if the reaction is allowed to run to completion, and the rate, specially the initial rate, of the production. These two quantities may be calculated from the formula $a = t/(p + bt)$ when the constants are known.

From an inspection of the curves it can be seen that as time proceeds the amount of carbon dioxide, called a, will approach a limiting value. Then, in the formula,

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[4] One source of error arises from the fact that different values for the constants will be obtained, depending on how much of the entire curve is used as a basis of calculation, and, as pointed out above, some of the experimental curves are incomplete. This error is accounted for, at least partially, by using only the first half of the complete curve; *i.e.*, the curve up to the point where one-half of the total amount of the carbon dioxide has been produced. The total amount is observed in some cases and estimated in others.

[5] The method of least squares is probably the most accurate method of determining the value of p and b, although any two points may be selected on a curve and the constants obtained by substituting observed values of a and t. For these curves the method outlined by Mellor (1909, p. 327) has been followed. Using the general equation $y = y/(a + bx)$ and a large number of points the following equations determine the constants:

$$a = \frac{\Sigma\,(xy)\,\Sigma\,(x^2y^2) - \Sigma\,(x^2y)\,\Sigma\,(xy^2)}{\Sigma\,(y^2)\,\Sigma\,(x^2y^2) - (\Sigma\,(xy^2))^2}$$

and

$$b = \frac{\Sigma\,(xy)\,\Sigma\,(xy^2) - \Sigma\,(x^2y)\,\Sigma\,(y^2)}{(\Sigma\,(xy^2))^2 - \Sigma\,(x^2y^2)\,\Sigma\,(y^2)}.$$

allow t to become very large. Since p is constant, t, and also bt, will become so large that p may be neglected. Then $a_{(\mathrm{lim})} = t/bt$. Cancelling t, $a_{(\mathrm{lim})} = 1/b$. The limiting value of a and the total amount of carbon dioxide (expressed in arbitrary units of course) will therefore equal $1/b$. This furnishes a convenient method for comparing the total amount of the action under different conditions.

In considering the rate of the activity we may deal with either a derivative or a tangent depending on whether we are considering an equation or a curve. With the experimental curves the tangents may be ascertained by means of instruments, and the rate curves then plotted if desired. The initial rate may be observed directly.

With the equation the procedure is different. If $a = t/(p + bt)$ then $da/dt = p/(p + bt)^2$. By substituting values for t the value of da/dt, or the rate, may be plotted. Now let t become exceedingly small compared with p, and the differential equation approaches the value $da/dt_{(\mathrm{lim})} = p/p^2$ or $1/p$. Therefore $1/p$ represents the initial rate of the production of carbon dioxide.

Applying these methods it is possible to get information concerning the concentration effects of the various reagents. Fig. 2 shows three curves obtained by plotting the values of $1/b$ against the concentrations of the reagents. Fig. 3 shows similar curves for the initial rates, as obtained from the equation and also from the tangents to the experimental curves.[6]

When the concentration of the pyrogallol or of the copper is varied we find that the limiting value of a varies as a constant fractional power of the concentration, or $a_{(\mathrm{lim})} = C^k$. This relation has been found very frequently in biological work, and owing to its similarity to the adsorption isotherm it has caused many phenomena to be ascribed to adsorptive processes. The present case makes it plain that adsorption cannot always be called upon to explain every process where the effect is proportional to some fractional power of the concentration. For here we have nothing but a chemical system. Pyrogallol and hydrogen peroxide do not show colloidal properties, and it is very unlikely that copper chloride exists in anything but an ionic or molecular form. Hence if adsorption is responsible for the fractional

[6] The figures obtained by these two methods are almost identical in their general character, the differences being due to the fact that different units are employed in the calculations. This close correspondence between the two sets of curves is evidence of the soundness of the method of calculation used in determining $1/p$. The values calculated for $1/b$ may also be accepted with confidence, since the manner of derivation is the same.

power relation then it must be in a sense very different from that commonly employed.

With hydrogen peroxide a curious relation comes to light. When its concentration is varied and the logarithm of the concentration is

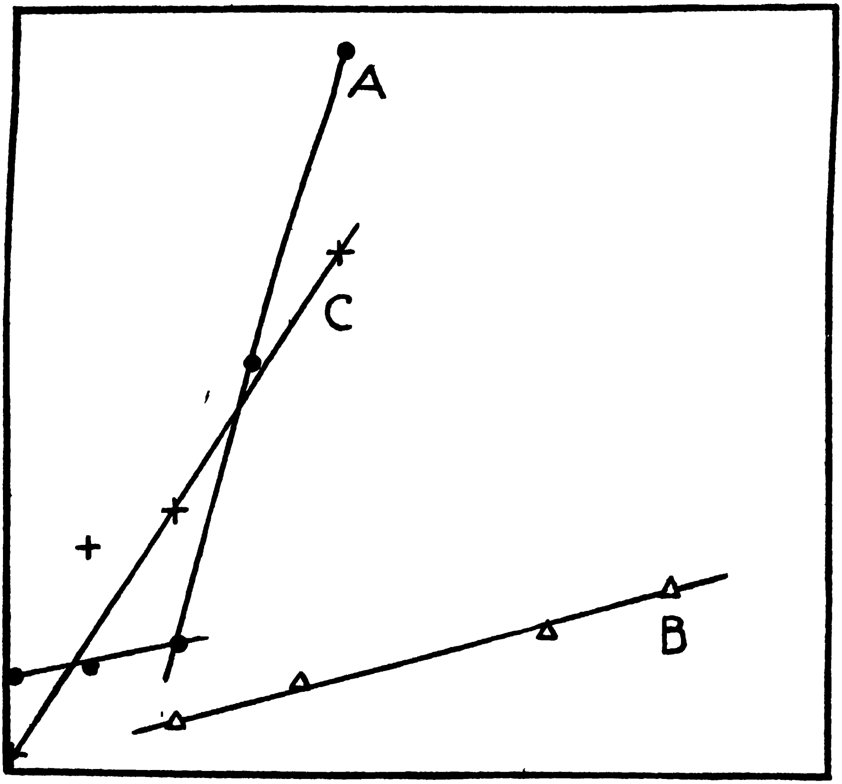

FIG. 2. Effect of concentration on the total amount of CO_2 produced. In all cases the ordinate is the logarithm of the amount of CO_2 as expressed by means of the logarithm of $1/b_{(\lim)}$ (see text). The abscissa is:

in Curve *A*, the logarithm of the concentration of $CuCl_2$;
" *B*, the logarithm of the concentration of H_2O_2 + the logarithm of the amount of CO_2;
" *C*, the logarithm of the concentration of the pyrogallol.

plotted against the ratio of the logarithm of the concentration to the logarithm of the effect a straight line is obtained. Expressed as an equation $\log a = \log C/(m + n \log C)$. That this relation has been previously observed may be shown by four instances where data from Snapper (1912), Dreyer and Walker (1914), Nothmann-Zuckerkandl (1912), and Plavec (1900), when plotted, give a straight line under similar conditions. However, the relation is unusual and no interpretation is available at present.

Fig. 2, Curve *A*, shows the effect of varying the concentration of copper. It will be noticed that while in general the law $a_{(\lim)} = C^k$ is obeyed, still, at 0.0001 M, there is a distinct break and the exponent k is different on each side. This is a situation which exists quite frequently in biological data. It might be maintained that at this critical concentration some change of phase relations occurs, such as the appearance of a new hydrate, dissociation product, or the like,

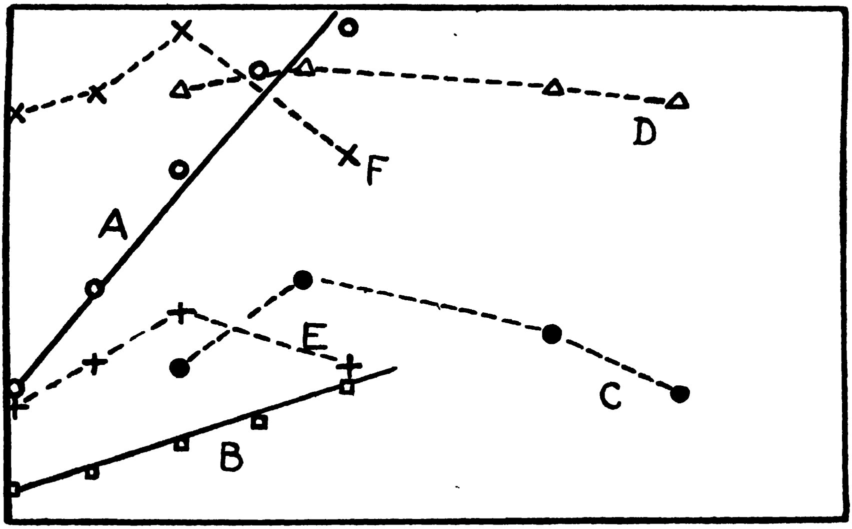

FIG. 3. Effect of concentration on the initial rate of reaction. In all cases the abscissa is the logarithm of the concentration. The ordinate in Curve *A* is the value of $1/p_{(\lim)}$ for $CuCl_2$;
in Curve *B*, the value of tangents to the beginning of experimental curves of $CuCl_2$;
" *C*, the value of $1/p_{(\lim)}$ for H_2O_2;
" *D*, the value of tangents with H_2O_2;
" *E*, the value of $1/p_{(\lim)}$ for pyrogallol;
" *F*, the value of tangents with pyrogallol.

which would suddenly alter the effective concentration of the copper. While it is impossible to offer an adequate and detailed explanation of this phenomenon, nevertheless certain interesting possibilities are suggested, particularly with regard to phase relations in biological experiments.

Fig. 3, Curve *A*, shows the effect of changing the concentration of copper on the initial rate of oxidation. Here the relation is da/dt (initial) $= k \log C$ or tan (initial) $= k \log C$. The lines are not perfectly straight, indicating some deviation from the simple law, but the fit is close enough to show that there is a continuous and definite variation of initial rate with concentration. On the other hand, there is no such clear-cut variation with the pyrogallol or peroxide. The variation, if any, is slight and seems to pass through a maximum. If three points can be said to determine a straight line in such a case then the relation is the same as that with copper, *i.e.*, da/dt (initial) $= k \log C$. But here there is a break where k changes from plus to minus. If the data cannot be said to warrant such a conclusion then the initial rate must be considered independent of the concentration and the apparent linear arrangement of the points purely fortuitous.

Whatever the interpretation of the data, the difference between copper and the other two reagents is striking. It suggests that the mode of action of copper is unique, and that it undergoes changes, or takes part in reactions, in which the other two constituents are not involved.

IV.

After copper the first metal investigated was iron. With hydrogen peroxide and pyrogallol it causes a rapid evolution of carbon dioxide. Contrary to what might have been expected, iron was but slightly more effective than copper. This doubtless is due, however, to the conditions existing in this particular system. Under other circumstances iron is a much more effective oxidation catalyst than copper. At the same time it should be borne in mind that as a toxic agent copper is far more powerful than iron.

The form of the time curve with iron was substantially the same as that with copper. The concentration effect was not investigated. One phenomenon worth mentioning appeared in connection with the experiments on iron. If several experiments were performed, using the same concentrations of all reagents, each experiment showed a larger production of carbon dioxide than the one preceding. This rendered it almost impossible to duplicate an experimental result. When the inside of the reaction chamber was coated with paraffin and the paraffin renewed occasionally, this difficulty vanished. The results became reproducible. Such a situation indicates that part of the iron was adsorbed or deposited on the glass wall of the tube and influenced subsequent reactions. No deposition of iron took place on the paraffin. Although no perceptible influence was ever exercised by the walls of the tube in the experiments on copper, paraffin was used as a safeguard.[7]

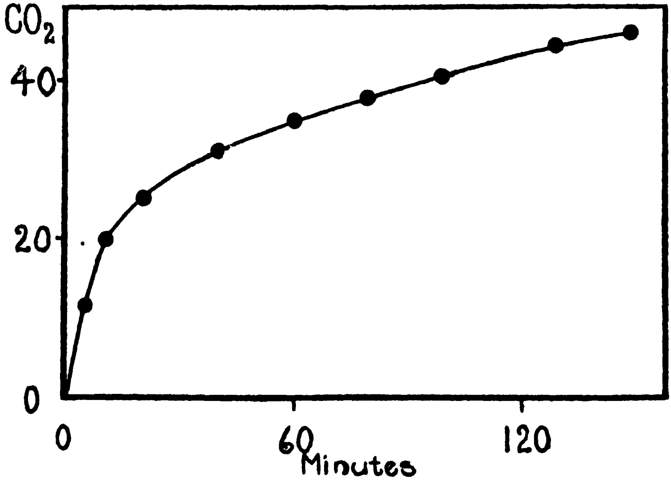

FIG. 4. Curve (average of three experiments) with $AuCl_3$, 0.01 M. The ordinate is the amount of CO_2 and the abscissa is time in minutes.

Several experiments were performed with silver nitrate and gold chloride. The action of these two elements being very similar only one, gold, will be discussed in detail. When the reaction with gold chloride starts, the production of carbon dioxide is relatively rapid (although not quite so rapid, for equivalent concentration, as with iron or copper). This period of great activity is followed by a much longer period of slight activity (see Fig. 4). The curve rises quite steeply for about 10 minutes, at the end of which time the rise becomes much more gradual. It appears as if there were two distinct curves, the first ending after about 10 minutes and merging into the second which continues for at least 2 hours. Examining the reagents it is found that the gold, which is introduced as a clear, yellow, solution of gold chloride, has precipitated as purple colloidal gold. If the reaction is followed in a test-tube it is observed that the precipitation begins after about 5 minutes and is apparently complete at 10 minutes. This precipitation corresponds in time to the sudden falling off in the rate of oxidation. Therefore the conclusion seems evident that the gold in solution catalyzes the oxidation of the pyrogallol to a marked extent, whereas the precipitated gold acts as a far less efficient catalyzer if indeed it catalyzes the reaction at all This matter will be discussed further in the next section.

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[7] There are numerous instances recorded concerning the effect of the glass wall of the containing vessel on chemical reactions.

Silver precipitates in a manner almost identical with that of gold, and the same considerations apply as with the latter metal.

Of several other metals none had a very marked effect. Cobalt and manganese caused a very slight production of carbon dioxide. They were each about as effective at a concentration of 0 01 M as copper at 0.00001 M; in other words, copper is about one thousand times as powerful a catalyst. Magnesium, mercury, cadmium, zinc, tin, and nickel had no detectable effect whatever. Hydrogen, in hydrochloric acid, was likewise without effect. The metals investigated may therefore be arranged in the following manner:

Group I: *Catalysts* (in order of effectiveness), Fe, Cu, Au, Ag, Co, Mn.

Group II: *Non-Catalysts*, Mg, Hg, Cd, Zn, Sn, Ni, H.

The theoretical significance of this grouping will be discussed later.

V.

When we come to consider the probable mechanism of this oxidation system two facts stand out as of primary importance:

1. All the experiments were performed in an acid medium. Hopkins (1925) has found in studying the mechanism of oxidation by means of glutathione that there is a marked difference in the course of the

reaction depending on whether the medium is acid or alkaline, and Meyerhof (1923) maintains that oxygen transfer by sulfhydril groups occurs only in acid and best at pH 3 to 5. (On catalysis by Fe in this connection, *cf.* Harrison, 1924.) These and other instances make it apparent that a change from an acid to an alkaline medium may profoundly alter the mechanism of an oxidation. Therefore it should be borne in mind in the discussion of the present data that all conclusions apply to experiments performed in an acid solution and that very different results might be obtained with experiments performed in an alkaline solution.

2. The experimental curves do not follow the course of a monomolecular or of a bimolecular reaction Since the possibility of a reaction of a higher order is remote, it is apparent that we have here a complex of reactions of different types. To construct a hypothetical arrangement which would duplicate all the experimental data would be very difficult, but a simple system can be set up which will give a general representation of what is found experimentally.

Let us consider the action of copper. There are two distinct phases to the oxidation of pyrogallol by this metal. First, the hydrogen peroxide must be decomposed in order to liberate the oxygen; and second, the oxygen must combine with the pyrogallol to produce carbon dioxide and other substances. Considerable research has been performed on the problem of the decomposition of hydrogen peroxide by metals. Bredig and Ikeda (1901) studied the decomposition of peroxide by platinum and considered that the action was due to the formation and reduction of an oxide of platinum. Oliveri-Mandalà (1920) found that a similar catalytic effect is produced by iridium, as had Bredig and Fortner (1904) with palladium. Berthelot (1901) found that silver forms a peroxide and afterward a superoxide with hydrogen peroxide, both of which decompose and liberate oxygen. Baeyer and Villiger (1901) started with the oxide Ag_2O and produced free oxygen with hydrogen peroxide. Colloidal silver was precipitated during the reaction., Hedges and Myers (1924) studied a periodic decomposition of hydrogen peroxide by silver, platinum, gold, and the enzyme catalase. These authors do not believe in the formation of an intermediate oxide.

In connection with the problem of "promoter action" the effect of copper and iron, as well as other substances, has been extensively investigated. Bray and Livingston (1923) worked with bromine. Their opinion is that the hydrogen peroxide is decomposed by the bromine molecule which is thereby ionized and which is later restored to the molecular state. The ion is therefore the intermediate product. Von Bertalan (1920)[8] says: "Die Zerzetzung des Wasserstoffperoxyds durch Eisenionen verläuft als eine typische monomolare katalytische Reaktion." He considers the ion as the effective catalyst. Mummery (1913)[9] says: "The catalytic decomposition of hydrogen dioxide by iron salts may be ascribed to the formation of higher perhydrols which are to be regarded as derivatives of hydrogen trioxide." Bohnson (1921) says that with iron the intermediate product is a hydrated ferric peroxide or "ferric acid." It is of interest that his data relating concentration to velocity constant (he finds the reaction to be of the first order) show breaks in continuity of very much the same sort as the break in Fig. 2, Curve *A*, where copper is the catalyst. Both ferric chloride and ferric sulfate exhibit this phenomenon. Bohnson also found that the specific reaction velocity decreases with time owing to the hydrolysis of the catalyst, and that free acid retards the action. Bohnson and Robertson (1923) conclude that the reaction, with iron, is an oxidation of the ferric ion, Fe, to a peroxide, FeO_4, and subsequent reduction to the ionic form. They base their opinion on thermodynamic reasoning. Goard and Rideal (1924) state that the intermediate compound varies according to the acidity of the solution; that in neutral solution it is Fe_2O_5 and in acid solution it is H_2FeO_4. Similarly, Spitalsky and Petin (1924) ascribe the activity of iron to intermediate products which vary in composition with the hydrogen ion concentration. Robertson (1925) has summed up the discussion and has given an explanation of the so called promoter action of metals. For the simple combination of copper and hydrogen peroxide he suggests the following equations:

$$Cu + H_2O_2 \rightarrow CuO_2 + 2\,H$$

$$CuO_2 + H_2O_2 \rightarrow Cu + 2\,H_2O + O_2$$

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[8] von Bertalan (1920), p. 328.

[9] Mummery (1913), p. 889.

The CuO_2 is copper peroxide and when in acid solution may exist as H_2CuO_3.

In view of the above accumulation of opinion and evidence it seems permissible to accept Robertson's equations as the basis for the first step in the oxidation of pyrogallol, and to emphasize the following three points:

1. The metal functions in the ionic form.
2. There is always an intermediate product formed.
3. This intermediate product is a peroxide.

This system applies when there is simply a metal and hydrogen peroxide present. When an oxidizable substance is added it is necessary to alter the supposed course of the reaction. The fact that hydrogen peroxide will color guaiac in the presence of a metal salt has long been known. A recent study is that of Aloy and Valdigué (1923) on copper. Karczag (1921) has investigated the oxidation of dyestuffs by metal salts and hydrogen peroxide. Another investigation bearing on the present problem is that of Bredig and Ikeda (1901), who found that many organic substances, including pyrogallol, "poisoned" the reaction between the metal and the peroxide.

When an organic substance such as pyrogallol is oxidized by means of this system one of two things must happen. Either the oxygen which is liberated from the hydrogen peroxide unites with the pyrogallol, or the oxygen is transferred directly from the metallic peroxide without any separate existence. The latter supposition is more probable and is entirely in line with current theories of oxidation. If we accept it then we must notice two facts:

1. The second stage of Robertson's reactions is altered because the oxygen in the CuO_2 does not escape as molecular oxygen but is transferred directly to the pyrogallol.

2. If this transfer released the ionic copper in its original state then the copper could go through the same cycle again and the reaction would continue until either the pyrogallol or the hydrogen peroxide was exhausted. But we know from experiments with low concentrations of copper that this is not the case. For instance, if we use 0.00001 M copper the production of carbon dioxide soon stops. If we then add more peroxide or pyrogallol there is no effect; but if we add more copper the oxidation begins again. This fact demonstrates

conclusively that the copper is steadily removed from the active state. The removal may take place in various ways but the simplest assumption is that the metal is bound up irreversibly with the pyrogallol. That some such process occurs is suggested by the results of Bredig and Ikeda (1901) on the "poisoning" effect of pyrogallol.

Let us call pyrogallol PC. Then

$$CuO_2 + PC \rightarrow CuP + CO_2$$

The carbon dioxide is measured and the inactive, or bound, copper remains in the solution. But we must complete the original equation. This we may do and write the entire scheme:

$$(1) \qquad Cu + H_2O_2 \rightarrow CuO_2 + 2\,H$$

$$(2) \qquad 2\,H + H_2O_2 \rightarrow 2\,H_2O$$

$$(3) \qquad CuO_2 + PC \rightarrow CuP + CO_2.$$

Of course this scheme may differ in detail from what actually takes place. The real reactions are doubtless more complicated than is indicated here. But it is possible to go a certain distance toward duplicating the experimental results by means of the theoretical equations. Considering reactions (1) and (2) it is evident that there is a bimolecular reaction between the copper and the hydrogen peroxide. But there are two molecules of hydrogen peroxide decomposed for every molecule of copper peroxide formed, owing to the reduction of the second molecule of hydrogen peroxide by the free hydrogen. Using the ordinary notation, if a is the initial concentration of copper, b the initial concentration of the hydrogen peroxide, and y the amount of copper peroxide formed after time t, then the rate of the reaction is proportional to the copper and hydrogen peroxide present. Of the latter, part is used in step (1) to form CuO_2 and H, and part in step (2) to form H_2O. These two parts are equal. Then we may write $dy/dt = K_1\,(a - y)\,(b - 2y)$. This equation may be integrated and expressed in the following form:

$$y = \frac{a\,b\,(1 - e^{K_1\,(a - b)\,t})}{b - 2\,a\,e^{K_1\,(a - b)\,t}}.$$

If x is the amount of carbon dioxide formed after time t, and c is the concentration of the pyrogallol, then x is proportional to the amount of pyrogallol and the amount of CuO_2 (or y) present after t units of time. This amount of CuO_2 is equal to the difference between the quantity produced from a and b and the quantity which has been transformed into x. Then $dx/dt = K_2\,(y - x)\,(c - x)$, or

$$x = \frac{y\,c\left(1 - e^{K_2(y-c)t}\right)}{c - y\,e^{K_2(y-c)t}}.$$

Since y is a variable quantity its value must be determined for every value of t and substituted in the above equation.

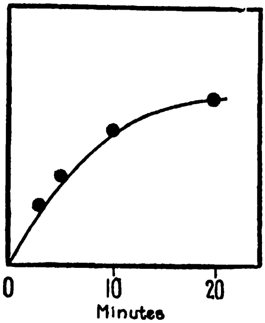

Fig. 5. The solid line represents a typical experimental curve ($CuCl_2$ 0.001 M H_2O_2 1 cc., pyrogallol 0.001 gm.). The points are obtained by means of the calculation outlined in the text whereby two bimolecular reactions are assumed. In this case $a = 0.5$, $b = 2$, $c = 0.08$, $K_1 = 0.2$, and $K_2 = 0.2$.

By the use of these equations the experimental curves may be approximately duplicated. Fig. 5 shows an example of an experimental curve selected at random and fairly closely fitted. The fit could be made much closer by more exact selection of the constants. This method of curve duplication does not afford absolute proof that the equations underlying the calculations are precisely those which underlie the experimental results. However, they must be fairly close to the truth. That the actual state of affairs is more complicated than has been suggested here is shown by one fact. In the calculated system the effect varies directly with the concentration, not as a fractional power thereof. In order to bring the calculated results into accordance with the experimental results it would be necessary to assume further complications such as side reactions, opposing reactions, *etc.* But the experimental data do not clearly indicate the precise nature of these additional reactions.

The définite conclusions which may be drawn are as follows: The oxidation of pyrogallol is due to the decomposition of the hydrogen peroxide by the copper ion, with the subsequent peroxidation of the copper. The copper peroxide then transfers its oxygen to the pyrogallol which is broken down into carbon dioxide and other products, including some form of combined copper.[10]

This system coincides with the Engler theory of respiration, in which there is an autoxidizable substance, a peroxide, and a catalyst. Atmospheric oxygen is absorbed and transferred by means of the peroxidase (catalyst) through the peroxide from the autoxidizable substance to the substance which is eventually to be oxidized. Here, of course, we start with a peroxide but the principle is the same. Wieland (1921) takes the view that hydrogen, not oxygen, is transferred, but he states that with metals and hydrogen peroxide metallic peroxides are formed. He therefore agrees in essentials with the intermediate peroxide theory.

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[10] The question of the atmospheric oxidation of pyrogallol and the nature of the products when the reaction is catalyzed by a metal has received attention from chemists. The two best known oxidation products are carbon dioxide and purpurogallin. The latter is specially likely to appear when iron is present. Salts of iron as well as of other metals may combine directly with pyrogallol, the reaction usually showing a characteristic color. Thus Jacquemin (1873) found that ferrous sulfate gives a blue color and ferric sulfate a red one.

de Clermont and Chautard (1882) got purpurogallin from pyrogallol in acid solution with $AgNO_3$ and $KMnO_4$. Wolff (1908) used ferrocyanide with hydrogen peroxide and obtained purpurogallin. Smirnow (1925) determined the effect of peroxidases by measuring the formation of purpurogallin, and found that manganese and iron catalyze the reaction although he does not consider that these two metals are necessary to a peroxidase. He quotes André (André, G., 1924, Chimie végétale, i, 210) to the effect that pyrogallol oxidizes according to the equation: $4\ C_6H_6O_3 + 9\ O \rightarrow C_{20}H_{16}O_9 + 4\ CO_2 + 4\ H_2O$ where $C_{20}H_{16}O_9$ represents purpurogallin. He also quotes Willstätter and Stoll (Willstätter, R., and Stoll, A., 1918, *Ann. Chem.*, cdxvi, 62), who state that $2\ C_6H_6O_3 + O_3 \rightarrow C_{11}H_8O_5 + CO_2 + H_2O$. The latter formula ($C_{11}H_8O_5$) is given as the correct formula for purpurogallin in Beilstein, F., 1923, Handbuch der organischen Chemie, 4th edition, vi, 1072. In the experiments here recorded the concentrations of the reagents were too low for noticeable quantities of purpurogallin to be produced.

VI.

Throughout the preceding discussion the assumption has been made that the metals act in the ionic form. This assumption is based on the results of Bohnson, Robertson, etc., according to whom the metals (in decomposing hydrogen peroxide) take part in ordinary chemical reactions. There is no reason to suppose that there is any change in the state of iron or copper when pyrogallol is present. Nevertheless there is evidence to show that certain metals in the colloidal state can also decompose hydrogen peroxide. Certain metals, furthermore, are precipitated in the colloidal form by pyrogallol. Bredig and Reinders (1901) decomposed hydrogen peroxide with colloidal gold, as did Galecki (1925). Bredig and Ikeda (1901) used colloidal platinum, Duclaux (1923) used colloidal iron, and Zenghelis and Papaconstantinos (1920) used colloidal rhodium. As to the effect of pyrogallol, in addition to calling attention to the reduction of silver bromide by pyrogallol in photography, reference may be made to Garbowski (1903), who precipitated gold, platinum, and silver, and to Henrich (1903), who precipitated gold, platinum, silver, and mercury with pyrogallol.

We have a very clear example of the different effect of ionic and colloidal metal in the experiments performed on gold which were outlined in a previous section. In these experiments we can watch the rate of oxidation change while the colloidal gold is being precipitated. The first few readings are obtained with ionic gold and show a rapid action. After the gold has been precipitated the action still continues but at a greatly reduced rate. There exist at this point two possibilities: either the gold is all precipitated and the slow action is due to the colloidal metal, or not all the gold is precipitated, the colloidal metal has no effect, and the slow rate is due to a very much reduced quantity of the ionic metal. To throw light on this matter a standard quantity of gold chloride was added in a test-tube to the usual amount of hydrogen peroxide and pyrogallol and after the gold had been precipitated the entire mixture was dialyzed for a week.

Then the colloidal gold was recovered, added to more hydrogen peroxide, and pyrogallol and the resulting action tested in the respiration machine. There was no detectable production of carbon dioxide. The conclusion follows that colloidal gold does not catalyze the oxidation of pyrogallol in measurable quantities, despite the fact that it decomposes hydrogen peroxide.

The question arises why colloidal gold does not oxidize pyrogallol. It will be remembered that to account for the action of copper it was assumed that the copper forms a peroxide which carries the oxygen from the hydrogen peroxide to the pyrogallol. It does not liberate molecular oxygen which then, of itself, combines with the pyrogallol. The formation of the intermediate peroxide is essential. Now it is clear that while ionic gold can react to produce a gold peroxide, the metal in the colloidal form cannot do so. Therefore as the gold in solution becomes aggregated in solid particles the concentration of the effective gold is reduced. That the ions are not completely eliminated and that all the gold is not changed into the colloidal form (as is demonstrated by the fact that some oxidation persists) is probably due to the fact that the solution is acid. That the degree of dispersion, size of particles, and general properties of colloidal metals are dependent to a large degree on the acidity of the medium has been shown by numerous investigators. The presence of the pyrogallol doubtless has considerable influence and may tend to prevent the complete precipitation of the gold in solution.

Obviously the mode of action of the metal in the colloidal state is very different from the mode of action in the ionic form. As the present case shows, ionic gold will catalyze the oxidation of pyrogallol while colloidal gold will not. But the latter will decompose hydrogen peroxide as well as the former. The decomposition, however, must be due to an entirely different mechanism in the two cases. The intermediate peroxide theory, which is very satisfactory with the metal in solution, offers no explanation whatever for the action of the colloidal metal since the colloid cannot be expected to enter into simple stoichiometric relations with the other constituents, including the formation of metallic peroxides. If, as has been assumed, the oxidation of pyrogallol depends on the presence of such peroxides, it is quite evident why colloidal gold can decompose hydrogen peroxide but fails to oxidize pyrogallol.

VII.

We are now in a position to make further comparison between the several metals which have been tested. A division has already been made between the effective catalysts, copper, iron, gold, silver, cobalt, and manganese, and the non-effective metals which include all the others tried. Among the first four there are marked differences depending on the ease with which the colloidal metal is precipitated. As a check on this point test-tube experiments were made which showed that gold and silver precipitated very easily, copper very slowly (a matter of days), and iron not at all. Iron is therefore the most powerful catalyst because it readily forms a peroxide and has the least tendency to be precipitated in the colloidal form by hydrogen peroxide and pyrogallol. Gold and silver are effective catalysts (though not so effective as iron and copper), but are prevented from exercising this function because they are so easily precipitated. Mercury, zinc, and the others, are ineffective because they do not form intermediate peroxides.

Herein may lie the reason why iron, and secondarily copper, are almost universally found in living cells as the metals which catalyze respiration. The assumption is legitimate that the cell contains organic peroxides similar to hydrogen peroxide and reducing (easily oxidizable) substances like pyrogallol. If this is true, and there is no strong evidence against it, then iron and copper are probably the only two metals which could effectively catalyze the oxidations in the cell. For metals like cobalt and manganese would be too weak in their action, mercury, zinc, etc., would not carry oxygen at all, and gold or silver would pass immediately into the ineffective colloidal state. Therefore iron and copper best fulfil the requirements for a metallic catalyst.

One final theoretical aspect of this question deserves brief consideration. If we examine the periodic table of the elements as revised to conform to the most recent ideas in physical chemistry, we find that all the active elements here investigated, *i.e.* iron, copper, gold, silver, cobalt, and manganese will be found close together in the table. Below is a reproduction of a portion of the table showing the arrangement of electrons in the atoms of the elements (according to Langmuir), found in Taylor's Treatise on physical chemistry (1924):[11]

	IIIa	IIIb	IVa
Group VII	Mn	—	—
Group VIII	Fe Co Ni	Ru Rh Pd	Os Ir Pt
Group I	Cu	Ag	Au
Group II	Zn	Cd	Hg

The groups refer to the standard groups in the periodic table. (The numerals at the head of the columns refer to the number of shells of electrons.) Thus we see that Group I, which includes copper, silver, and gold, has an outer shell of one electron, and Group VIII has an outer shell which lacks completeness by one electron. It is in these two groups that the effective elements Fe, Cu, Ag, Au, and Ru, Rh, Pd, Os, Ir, and Pt, which are probably also effective, occur. The only exceptions are manganese which is in Group VII, and nickel which is in Group VIII but is not effective. In general the situation is striking. Those elements which are effective oxidation catalysts have their outer shell either composed of just one electron or lacking just one electron. Furthermore, as the number of shells increases the greater becomes the tendency for the element to pass into the colloidal state with hydrogen peroxide and pyrogallol. It is impossible to draw any conclusions at present from these facts, but they are very suggestive and indicate the possibility of future theoretical developments.

SUMMARY.

1. When iron and copper are allowed to act on hydrogen peroxide and pyrogallol, enough carbon dioxide is produced to be readily measured.

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[11] Taylor (1924), p. 1062.

2. The curve of the production of carbon dioxide may be fitted by an empirical equation, by the use of which the initial rate and the total amount of the oxidation may be determined.

3. The effect of the concentration of the reagents is different in each case, the effect varying as a fractional power of the copper and pyrogallol concentrations and as a logarithmic function of the hydrogen peroxide concentration.

4. When gold or silver is used the rate changes suddenly during the course of the reaction due to the precipitation of colloidal metal.

5. Mercury, cadmium, zinc, tin, and some other metals have no effect.

6. A theoretical set of equations is assumed to account for the action of the metals.

7. The metals are assumed to act by means of the formation of intermediate peroxides.

8. Experiments on the action of gold indicate that the metals are active in the ionic and not in the colloidal state.

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AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

I. THEORETICAL.*

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The attempts to explain a part of the behavior of living cells or tissues on the assumption that they act as simple ampholytes, while fairly satisfactory and suggestive, must be considered only as a first suggestion. Those who employ this concept admit that living cells are more complicated systems than simple proteins, so that clear-cut results from studying them from such a point of view are not to be expected. Certain recent researches cannot, however, be easily correlated on the basis of mere lack of "clear-cutness." For example, Robbins (1), from the staining reactions and water absorption of potato tuber, has shown that it acts as an ampholyte with an isoelectric point at a pH of about 6. However, Cohn, Gross, and Johnson (2) have found that the typical potato protein, tuberin, obtained by acid precipitation of potato juice, has an isoelectric point at a pH of about 4.

Winslow, Falk, and Caulfield (3) have studied the electrophoretic behavior of the organism *Bacillus cereus* over a wide pH range, and while, in the main, the curve obtained may be explained on the basis of a simple Donnan equilibrium, there is a comparatively wide pH range through which such an explanation cannot hold.

Certain unicellular organisms seem to show little tendency to retain either acid or basic dye, through a comparatively wide pH range, in place of through only a narrow range as might be expected to be characteristic of the isoelectric behavior of a simple ampholyte (4). Other organisms show no point where combination with one or the other type of dye does not seem to take place to a considerable extent.

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* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 115.

It is the purpose of this paper to examine the probable behavior of a system of two amphoteric substances between which mutual combination may take place under proper conditions, and to show that, by employing the considerations involved, it is much easier to explain much of the physical and chemical behavior of living tissues than it is by using the concept of a simple ampholyte. In the two following papers experimental evidence is adduced, from a study of certain simple systems of two ampholytes, in support of this idea. It may, however, be pointed out that living cells are by no means as simple as this above concept would seem to indicate. Its justification lies in the fact that, by sacrificing only very little of the simplicity of treatment which suffices for consideration of simple ampholytes, one gains greatly in comprehensiveness.

Concept of a Conjugate Protein.

Consider an aqueous solution of two amphoteric substances, HAOH with ionization constants K_a and K_b and an isoelectric point at a hydrogen ion concentration I, and HBOH with corresponding constants K'_a and K'_b and an isoelectric point at I'. Suppose I is larger, *i.e.* more acid, than I'.

In solutions whose hydrogen ion concentration is appreciably greater than I both components will act as bases and tend to retain acids. When the hydrogen ion concentration is appreciably less than I' both components will act as acids and tend to retain bases. In solutions whose hydrogen ion concentration lies between I and I', however, one component will act as a base and the other as an acid, and there will be a tendency toward mutual combination, resulting in a decreased retention of acid or basic reagent. This pH range, from I to I', is the range of mutual combination; and the stability of the system, so far as its behavior as a distinct individual is concerned, depends among other things on the magnitude of this range. This might explain, for example, why certain conjugated proteins, such as lecithoproteins, though they are thought to exist, have not been definitely isolated, while others such as nucleoproteins or phosphoproteins can be easily obtained.

Apparent Isoelectric Behavior.

In a system of two ampholytes we have the following equilibria:

$$\overset{+}{\mathrm{H}} + \mathrm{A}\overset{-}{\mathrm{OH}} \rightleftarrows \mathrm{HAOH} \rightleftarrows \mathrm{H}\overset{+}{\mathrm{A}} + \mathrm{O}\overset{-}{\mathrm{H}} \text{ with constants } K_a,\ K_b, \text{ and } I;$$

and

$$\overset{+}{\mathrm{H}} + \mathrm{B}\overset{-}{\mathrm{OH}} \rightleftarrows \mathrm{HBOH} \rightleftarrows \mathrm{H}\overset{+}{\mathrm{B}} + \mathrm{O}\overset{-}{\mathrm{H}} \text{ with constants } K'_a,\ K'_b, \text{ and } I'.$$

If HAOH is the more strongly acidic, there will be an isoelectric point at the pH at which

$$(\mathrm{A}\overset{-}{\mathrm{OH}}) + (\mathrm{B}\overset{-}{\mathrm{OH}}) = (\mathrm{H}\overset{+}{\mathrm{A}}) + (\mathrm{H}\overset{+}{\mathrm{B}})$$

The value of the hydrogen ion concentration corresponding to this point is obtained by expressing the above quantities in terms of $(\overset{+}{\mathrm{H}})$ and the various constants and solving. We obtain

$$(\overset{+}{\mathrm{H}}) = \sqrt{K_w \frac{K_a\,(\mathrm{HAOH}) + K'_a\,(\mathrm{HBOH})}{K_b\,(\mathrm{HAOH}) + K'_b\,(\mathrm{HBOH})}} \qquad (1)$$

The isoelectric point is not exactly the point of maximum mutual combination. The latter point may be expected to be governed by the condition

$$(\mathrm{A}\overset{-}{\mathrm{OH}}) = (\mathrm{H}\overset{+}{\mathrm{B}})$$

and will occur at a hydrogen ion concentration obtained from the following expression:

$$(\overset{+}{\mathrm{H}}) = \sqrt{K_w \frac{K_a\,(\mathrm{HAOH})}{K'_b\,(\mathrm{HBOH})}}$$

The two points, though not identical, will lie very close together; and the simple expression may be used for calculating the isoelectric point of the system.

Electrophoretic Behavior.

From the amphoteric equilibria given above we can, by applying the mass-action law and differentiating with respect to the logarithm of the hydroxide ion concentration, obtain the following four equations:

$$\frac{d(\mathrm{AO\overset{-}{H}})}{d\,ln(\mathrm{O\overset{-}{H}})} = \frac{K_a\,(\mathrm{HAOH})\,(\mathrm{O\overset{-}{H}})}{K_w}. \tag{2}$$

$$\frac{d(\mathrm{H\overset{+}{A}})}{d\,ln(\mathrm{O\overset{-}{H}})} = -\frac{K_b\,(\mathrm{HAOH}).}{(\mathrm{O\overset{-}{H}})} \tag{3}$$

$$\frac{d(\mathrm{BO\overset{-}{H}})}{d\,ln(\mathrm{O\overset{-}{H}})} = \frac{K'_a\,(\mathrm{HBOH})\,(\mathrm{OH})}{K_w}. \tag{4}$$

$$\frac{d(\mathrm{H\overset{+}{B}})}{d\,ln(\mathrm{O\overset{-}{H}})} = -\frac{K'_b\,(\mathrm{HBOH})}{(\mathrm{O\overset{-}{H}})}. \tag{5}$$

from which

$$\frac{d(\mathrm{AO\overset{-}{H}})}{-\,d(\mathrm{H\overset{+}{B}})} = \frac{K_a\,(\mathrm{HAOH})\,(\mathrm{O\overset{-}{H}})^2}{K'_b\,(\mathrm{HBOH})\,K_w}$$

or, since

$$(\mathrm{O\overset{-}{H}}) = K_w/(\overset{+}{\mathrm{H}}),$$

$$d(\mathrm{AO\overset{-}{H}}) = -\frac{K_a\,(\mathrm{HAOH}\,(\mathrm{O\overset{-}{H}})}{K'_b\,(\mathrm{HBOH})\,(\overset{+}{\mathrm{H}})}\,d(\mathrm{H\overset{+}{B}})$$

Either an increase in $(\mathrm{AO\overset{-}{H}})$ or a decrease in $(\mathrm{H\overset{+}{B}})$ will increase the resultant negative charge on the micella, and thus increase the velocity toward the anode in a constant electric field. At the isoelectric point, as the hydroxide ion concentration is increased, $\frac{d(\mathrm{AO\overset{-}{H}})}{d\,ln(\mathrm{O\overset{-}{H}})}$ is of the same order of magnitude as $\frac{-\,d(\mathrm{H\overset{+}{B}})}{d\,ln(\mathrm{O\overset{-}{H}})}$ but the former increases with increasing alkalinity while the latter decreases (equations (3) and (4)). Through the pH range I to I', the value of $-d(\mathrm{H\overset{+}{A}})$

is small compared to that of $d(\mathrm{AO\overset{-}{H}})$, and $d(\mathrm{BO\overset{-}{H}})$ is small compared to $-d(\mathrm{H\overset{+}{B}})$. As a result there will be a rather rapid increase in negative charge through a certain range, passing through the isoelectric point, until nearly all the HAOH is ionized to $\mathrm{AO\overset{-}{H}}$, which ionization takes place increasingly rapidly as $(\mathrm{O\overset{-}{H}})$ is increased. When such a condition is reached, the only possible significant increase in negative charge before the point I' is reached is from $-d(\mathrm{H\overset{+}{B}})$, which has less and less effect on the magnitude of the charge as $(\mathrm{H\overset{+}{B}})$ becomes smaller, *i.e.* as $(\mathrm{O\overset{-}{H}})$ is increased (equation (4)). This means that through a certain pH range the negative charge will remain nearly constant. When I' is reached, however, we have $\dfrac{-d(\mathrm{H\overset{+}{B}})}{d(\mathrm{BO\overset{=}{H}})} = 1$, and from this point on $\dfrac{d(\mathrm{BO\overset{-}{H}})}{d\,ln(\mathrm{O\overset{=}{H}})}$ is the predominant factor. Its value increases with $(\mathrm{O\overset{-}{H}})$, and the negative charge again begins to increase more rapidly, and continues until the HBOH is completely ionized. From this point on, since we are now on the alkaline side of the isoelectric points of both components and they are thus both in the same ionic state, we may expect the curve to be the same as would be predicted by the application of the Donnan equilibrium to a simple ampholyte.

A similar condition would prevail on the acid side of the isoelectric point of the system as the hydrogen ion concentration is increased.

DISCUSSION.

It may be well to cite some of the observations which originally led to the more definite formulation of the concept of a mixed amphoteric system.

In connection with certain bacteriological problems the staining reactions of a large number of organisms have been studied by the author (4). Certain of the typical curves are given in Fig. 1.[1] Abscissæ are pH values and ordinates are arbitrary functions of the intensity of retained color. Values for the latter were obtained by repeated comparison of slides under the microscope, and are there-

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[1] Bacterial cells furnish a very satisfactory material to study. The individual cell as a system can be easily observed, thin smears can be obtained fairly free from débris, and equilibrium can be quickly reached. We found little difference in results between buffering for several minutes and for as long as 150 hours. Germ-

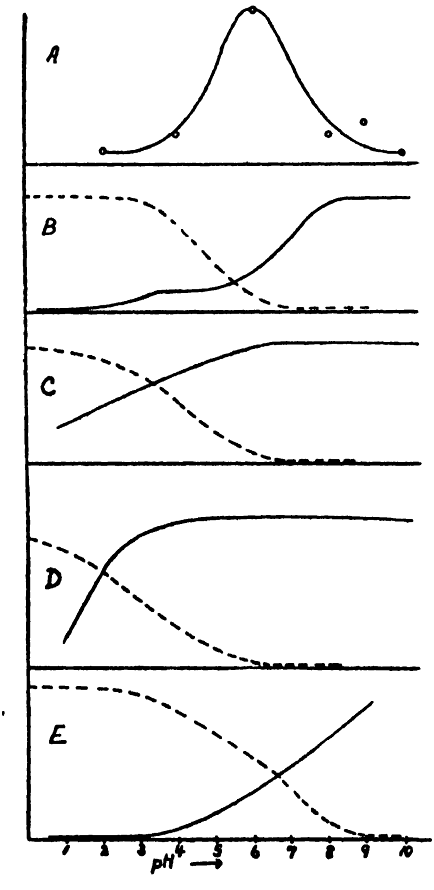

FIG. 1. For explanation see text. Broken lines represent behavior toward acid fuchsin, unbroken lines (except Curve A) behavior toward gentian violet.

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gross, along the same line (6), states that hide powder in small quantities, unhardened by formaldehyde, reaches complete reversible equilibrium with acid solutions in 2 minutes. Our final technique consisted in preparing thin smears on microscope slides, staining with "carbol gentian violet" or "carbol acid fuchsin" solution, "fixing" by treatment with buffer solution, and finally decolorizing with acetone. Slides of any series were then repeatedly compared under a microscope.

fore only qualitative. They are, however, comparative and the curves represent the behavior of the organism. The intensity plotting has been conservative and quantitative methods would, we feel sure, merely accentuate the contrast between the greater and smaller dye retention.

In Curve A of this figure the buffer ratios for the organism *Bacillus coli*, obtained by Falk and Shaughnessy (5) are plotted just above the color curve for the same organism (Curve B).[2] The results are suggestive, showing that the buffering power of this organism shows itself over a wide pH range with a maximum near the isoelectric point of the system as determined by minimum dye retention.

The curves in this figure are typical of classes of bacteria. A fair number of organisms have their point of least color retention around a pH of about 3 (Curve D), another large class has a corresponding point around a pH of 5 to 6 (Curve B), and there are a few intermediate (Curve C).

Other types of cells were studied, such as red blood cells from both human blood (Curve E) and sheep blood. In the case of the former the smears were made from whole blood and the isoelectric point of such a system lies a little below a pH of 7. The sheep cells used were washed cells and showed a corresponding point at a pH very near 7.

Such a system as *Bacillus coli* (Curve B) exhibits a range through which little dye is retained—either acid or basic—suggesting a considerable stability of the system as a chemical individual, while the system *Bacillus dysenteriæ* Shiga (Curve C), even at its isoelectric point, still retains fairly strongly both acid and basic dyes, showing that there is still a fair concentration of both cation and anion in the system, and that either actual combination between these two is limited, or that the combination is comparatively unstable.

The condition of maximum combination between the two components of a system of two ampholytes need not mean a condition of much combination. When $(\text{AO}\overset{-}{\text{H}}) = (\text{H}\overset{+}{\text{B}})$, though this is the optimum condition for combination, the system may act as a majority of ionogens and remain largely in ionic form. On the other hand it may act in a manner analogous to such salts as lead acetate, mercury salts, etc., and the two ions may almost entirely combine. The extent of this combination will determine the behavior toward anions and cations. In systems in which the two components are largely combined at the isoelectric point we may have behavior simulating a simple ampholyte in that there will be a pH range through which no appreciable combination with added cation or anion takes place.

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[2] In plotting Curve A the pH range given in the table by Falk and Shaughnessy is plotted at its lowest value, thus where they give the range 6–7, it is plotted as 6.

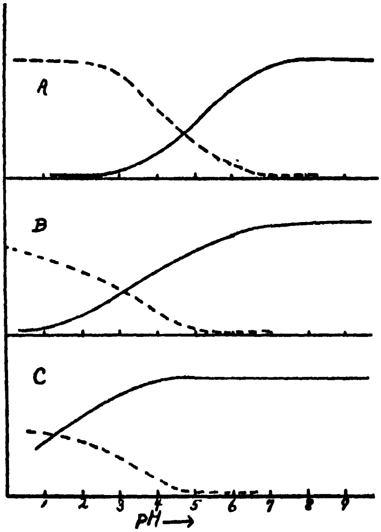

FIG. 2. Effect of oxidation on the behavior of *Bacillus typhosus* toward acid fuchsin (broken line) and gentian violet (unbroken line). Curve A—original organism, Curve B treated with N/50 iodine, Curve C treated with N/50 potassium dichromate.

On the other hand, we may have systems in which even at the isoelectric point there is still a fair concentration of both $(\mathrm{AO}\overset{-}{\mathrm{H}})$ and $(\mathrm{H}\overset{+}{\mathrm{B}})$ and thus there will be no point at which added anions and cations will not be appreciably bound.

There are at least two ways in which the isoelectric point of a mixed system or of a simple ampholyte may be changed. The system may be transformed into a new mixed system either by altering the relative amounts of the components or by adding another component, or the acid or basic properties may be altered by oxidation or reduction.

An example of the first effect is reported by Gerngross (7) who found that the electrophoretic isoelectric point of gelatin was changed from a pH of 4.75 to 4.3 by treatment with formaldehyde.

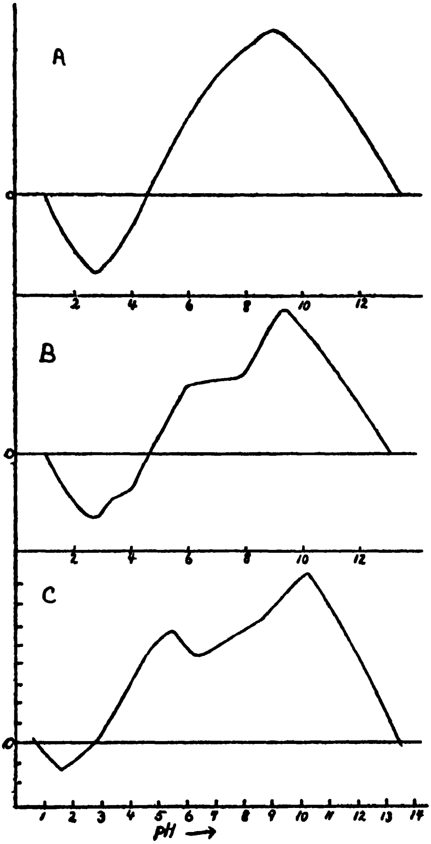

FIG. 3.

Change in isoelectric point by oxidation has been studied by the author (4), and certain results are shown in Fig. 2. The magnitude of the change depends on the degree of oxidation. A mild oxidizing agent such as iodine (Curve B) renders the system more acidic than it was originally (Curve A), but such an oxidizing agent as potassium bichromate produces a distinctly greater shift in the same direction (Curve C).[3]

While reducing agents do not seem to have any effect on the original organism, the effect produced by oxidation has been repeatedly reversed by treatment with stannous chloride. This behavior is, of course, analogous to the behavior of practically all substances in the effect of oxidation or reduction on acidic strength.

An interesting application of the concept of a mixed system is the electrophoretic behavior of the organism *Bacillus cereus* as worked out by Winslow, Falk, and Caulfield (3) and by Winslow and Shaughnessy (8). Their results are roughly represented by Curve C of Fig. 3. Abscissæ are pH values and ordinates are migration velocities in an electric field. These are measured toward the anode on that portion of the curve above the zero line and toward the cathode below this line. Curves A and B are both theoretical. Curve A represents the theoretical electrophoretic behavior of a micella composed of a single ampholyte to which the Donnan equilibrium applies. Curve B represents the theoretical electrophoretic behavior of a system of two ampholytes as worked out above. The intersection of the curves with the zero line represents the isopotential point.

In all the cases mentioned above the concept of a mixed system of ampholytes, so simple as to contain only two components, offers a much more obvious explanation of the experimental facts than the concept of a simple ampholyte.

SUMMARY.

The amphoteric behavior of a system of two amphoteric components is theoretically examined; and this is shown to correspond more nearly with certain of the physical and chemical behaviors of living tissues than does the concept of a simple ampholyte.

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[3] Oxidizing agents were incorporated in the buffer solutions in N/50 concentrations.[1] A goodly number of oxidizing agents were studied, and, in general, they could be arranged in a series on the basis of the magnitude of their effect on the organisms, which series was roughly the same as arrangement on the basis of oxidizing potential. The increase in acid properties upon treatment with an oxidizing agent, illustrated in Fig. 2, was noted in all organisms studied.

It is a pleasure to acknowledge the criticism and suggestions of Dr. A. L. Raymond in the preparation of the manuscript of this and the three following papers.

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AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

II. Titration of Sulfanilic Acid-Glycine Mixtures.*

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(Accepted for publication, July 26, 1926.)

The considerations presented in the preceding paper were developed as an attempt to explain results obtained in studying living cells (1). It is desirable to test them on much simpler systems. Michaelis and Davidsohn (2), in some flocculation experiments, obtained data which support the above ideas in a qualitative way. They state that, if two amphoteric colloids are mixed, a combination may precipitate out whose flocculation optimum lies between their respective isoelectric points. Thus with nucleic acid and denatured serum albumin, with isoelectric points of 2×10^{-1} and 4×10^{-6} respectively, there is a combination with optimum flocculation at a hydrogen ion concentration of 1.6×10^{-4}.

It is the purpose of this and the following paper to test portions of the theory in a more quantitative manner, and in this paper the mutual action of two ampholytes, glycine and sulfanilic acid, is studied by means of titration over a wide pH range. The latter substance was chosen to correspond to the strongly acid nucleic acid in living matter. That it is amphoteric, with a measurable basic ionization constant, is shown in the fourth paper of the series. In fact it has an isoelectric point at a distinctly higher pH, 1.25, than that (0.7) of nucleic acid (2). The glycine used was a preparation from the Eastman Kodak Company; and the sulfanilic acid was a Merck c.p. grade, which was reprecipitated from alkaline solution by hydrochloric acid, washed, and dried.

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*Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 116.

Method.

An ordinary potentiometer set up sensitive to 0.25 millivolt, was employed. The hydrogen electrodes were of the type described by Bailey (3). A saturated calomel electrode (4) was used in connection with a saturated KCl bridge.

A series of solutions of hydrochloric acid and carbon dioxide-free sodium hydroxide was made up, each solution of definite known concentration. Points on the titration curve were obtained by introducing 15 cc. of one of these solutions into a small glass-stoppered bottle with 10 cc. of a standard solution of the substance to be titrated, and the equilibrium hydrogen ion concentration of the resulting mixture determined. The difference between the normality of the acid or base diluted with 10 cc. of water and that diluted with 10 cc. of the glycine or the sulfanilic acid will give the amount neutralized. The original normality, N, is known and the latter can be obtained from the measured cH if we know the degree of ionization, *i.e.* the normality is equal to cH/α, where α is the degree of ionization, which value

TABLE I.

HCl				NaOH			cOH
N	E.M.F.	pH	cH	N	EMF	pH	
	mv				*mv*		
001	426	3 00	001	004	924 5	11 425	00266
003	399	2 533	00293	009	943	11 74	0055
006	380	2 217	00607	015	958	12 00	010
009	370	2 050	00891	025	968 5	12 175	015
012	363	1 933	0117	040	981 7	12 390	0246
018	353	1 760	0174	060	990 3	12 540	0347
025	345 5	1 625	0237	080	997 5	12 660	0458
040	333 5	1 425	0376	100	1001 5	12 730	0538
060	323	1 250	0562	130	1008 5	12 860	0720
090	313	1 083	0826	180	1014 8	12 960	0916
.120	305	0 950	1122	250	1024	13 117	1310
.150	300 5	0 875	1334	3511	1030	13 217	165
.200	292 5	0 742	181				
250	287 8	0 663	217				
320	282	0 56	2754				

must be determined *potentiometrically* (5). Thus the number of mols, *n*, of the HCl or the NaOH neutralized by the glycine or sulfanilic acid is given respectively by the expressions:

$$n = \text{N} - \frac{cH}{\alpha} \text{ for HCl; and } n = \text{N} - \frac{cOH}{\alpha} \text{ for NaOH}$$

The only assumption involved is that at the same normality the acid

TABLE II.

N HCl	E.M.F.	pH	cH	*n*
	mv.			
.320	285.	0.617	.2415	.041
.250	291.8	0.730	.1862	.0395
.200	298.5	0 842	.1439	.040
.150	307.5	0 992	.1019	.0390
.120	316.	1 133	.0736	.0405
.090	326 5	1.308	.0492	.0375
.060	344.5	1 608	.02466	.0345
.040	365.3	1.970	.01072	.0291
.025	388.	2.350	.00477	.0205
.018	400.5	2 558	.00277	.0152
.012	414	2.800	.00159	.0104
.009	422.5	2.942	.00114	.00786
.006	434.	3 133	.000736	.00526
.003	455.5	3.492	.000322	.00268
.001	484.	3.98	.000105	.0009
.0000	598.	5.9	—	—
NaOH			cOH	
.004	766.	8.75	.0000056	.004
.009	790.	9.15	.000014	.009
.015	806.5	9.43	.000027	.015
.025	831.5	9.86	.000073	.0249
.0373	871.5	10.53	.00034	.0368
.040	895.	10.933	.00085	.0387
.060	964.	12.10	.01259	.040
.080	981.	12.383	.02415	.0397
.100	990.5	12.542	.03483	.0401
.130	1000.5	12.708	.05105	.0397
.180	1009.5	12.875	.0750	.0398
.250	1019.	13.033	.1079	.0405
.3511	1028.	13.183	.1524	.040

or alkali ionizes to the same extent in the presence of the glycine or sulfanilic acid as it does when these are absent.

Table I gives values of cH and cOH for various normalities of HCl and NaOH at room temperature, 20–22°C. By plotting N against cH or cOH, curves are obtained by means of which the normality of acid or base corresponding to any hydrogen or hydroxyl ion concentration may be read.

TABLE III.

Sulfanilic acid.				Glycine.			
0.04 N		0.023 N		0.10 N		0.02 N	
pH	*n*	pH	*n*	pH	*n*	pH	*n*
				1.14	.097	0.913	.020
				1.483	.0864	1.033	.019
				2.26	.0544	1.183	.0194
				2.56	.0372	1.408	.0186
				2.86	.0236	1.638	.016
				3.033	.0178	2.01	.015
				3.233	.0142	2.192	.0115
				3.37	.0086	2.467	.0086
				3.55	.0057	2.622	.0066
				3.85	.00286	2.867	.00464
						3.20	.00237
						3.70	.0008
2.13	—	2.47	—	5.9	—	5.9	—
2.483	.004	3.033	.009			9.533	.00845
2.683	.009	3.50	.015			10.017	.0140
2.917	.015	3.54	.018			10.05	.0164
3.282	.025	11.18	.023			11.45	.0197
4.50	.040	12.03	.0226			12.07	.020
12.084	.0405	12.33	.0234			12.36	.0203
12.367	.0405						
12.553	.040						
12.70	.039						
12.88	.039						
13.033	.0395						
13.183	.04						

Titration of Glycine and of Sulfanilic Acid.—An N/10 solution of glycine was prepared, and 10 cc. of this added to 15 cc. each of the various solutions of HCl and NaOH. The resulting glycine concentration was thus 0.04. Table II gives the results with glycine. N is the original normality of the HCl or NaOH after dilution from 15 to 25 cc., and *n* is the number of mols neutralized by the glycine, per liter of mixture.

TABLE IV.

0.04/0.043		0.02/0.02		0.10/0.04		0.04/0.08	
pH	*n*	pH	*n*	pH	*n*	pH	*n*
0.617	.041	1.033	.019	1.467	.0841	1.525	.0286
0.73	.0395	1.18	.0194	2.175	.0532	1.825	.0246
0.84	.04	1.41	.0170	2.433	.0363	2.117	.01724
0.99	.039	1.62	.015	2.625	.0226	2.208	.01174
1.125	.039	1.97	.014	2.73	.01614	2.333	.0092
1.31	.0375	2.15	.0108	2.833	.011	2.39	.0049
1.58	.033	2.32	.00715	2.88	.0077	2.48	.0027
1.91	.0273	2.405	.00506	2.97	.0049	2.55	.00017
2.21	.0186	2.525	.00301	3.017	.00204		
2.36	.0136	2.65	.00076				
2.48	.0087						
2.575	.00634						
2.625	.00363						
2.683	.00092						
2.74	—	2.76	—	3.06	—	2.60	—
2.8	.001	2.967	.004				
2.85	.003	3.217	.009				
2.933	.0075	3.583	.015				
3.083	.0133	8.867	.025				
3.483	.0266	10.38	.0397				
4.11	.039	12.067	.041				
4.517	.0412	12.367	.041				
6.14	.0433	12.55	.039				
8.25	.045	12.71	.0397				
8.70	.048	12.883	.039				
9.175	.054	13.03	.0405				
9.45	.060	13.183	.040				
10.15	.0748						
10.92	.082						
12.05	.0829						
12.32	0824						

Table III gives, in condensed form, results obtained with certain concentrations of sulfanilic acid as well as certain other concentrations of glycine. The solubility of the sulfanilic acid prevented making an N/10 solution at room temperature, so the solution was made up at 40°C. using such a volume that 0.1 mol would occupy a volume of 1 liter at 20°C. A quantity was pipetted from this solution at 40° such that it would occupy a volume of 10 cc. at 20°. As may be expected, acid titration of sulfanilic acid has little meaning, since at the pH where any effect may be expected, a very small change in E.M.F. corresponds to a large quantity of sulfanilic acid neutralized. Thus, except for glycine, only alkaline titrations are included.

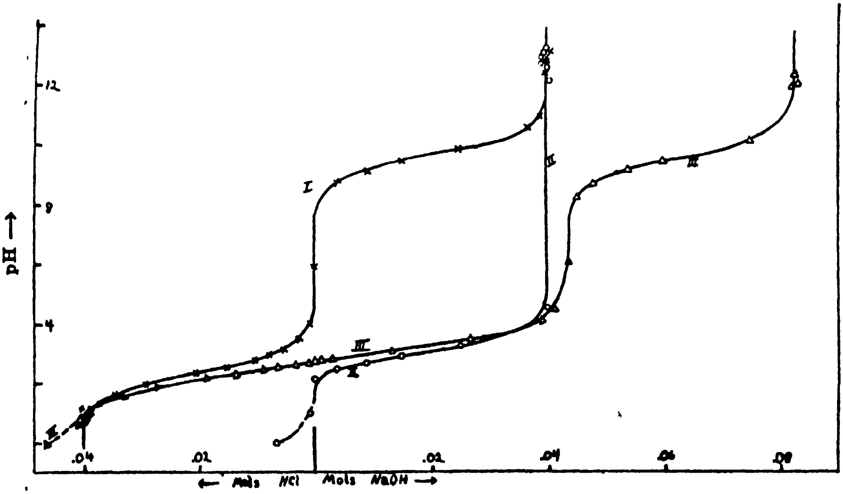

FIG. 1.

In Tables III and IV, *n* represents mols of HCl neutralized when it is above the center line, and mols of NaOH neutralized when below this line.

Table IV, which is analogous to Table III, gives results obtained from the titration of various mol ratios of glycine and sulfanilic acid. The concentration of glycine is given first in the ratios, *i.e.* the ratio 0.04/0.043 means a mixture containing 0.04 mol glycine and 0.043 mol sulfanilic acid per liter.

The curves in Fig. 1 are plotted from data obtained by titration of the 0.04 N glycine (Curve I), the 0.04 N sulfanilic acid (Curve II), and the 0.04/0.043 mixture of glycine and sulfanilic acid (Curve III). The alkaline titration of the sulfanilic acid (Curve II) is plotted on an abscissæ scale 43/40 that of the other curves to compare its behavior easily with that of the mixture. The broken portions of the curves are drawn, not through directly determined experimental points, but through points calculated from the basic ionization constant of sulfanilic acid (6). Abscissæ give the mols of HCl, measured to the right of the zero line, or of NaOH, measured to the left of this line, neutralized, as calculated by the formula given above, while ordinates give the corresponding pH. Similar curves may be obtained by plotting in a like manner the data from the other titrations.

The striking thing about the curve is the high buffering action of the mixture of glycine and sulfanilic acid about a pH which, it will be shown, corresponds closely with the value that one would calculate for what has been termed the isoelectric point of the system, from the formula developed in the first paper of this series. Moreover, it will be noted that the behavior of the mixture as a seeming individual, at least with a characteristic curve differing from the curves of the components studied alone, is confined to that portion of the pH range between the respective isoelectric points of the components. At a pH above the isoelectric point of glycine the curve representing the behavior of the mixture is an exact duplicate of the glycine curve, displaced to the right, of course, by the amount of the sulfanilic acid concentration in the mixture. In the same way there is no reason to doubt that the curve below the isoelectric point of the sulfanilic acid, at a pH of 1.25 (6), and that of the pure sulfanilic acid would also duplicate each other, though unfortunately, with this substance, that portion of the curve cannot be easily experimentally realized.

Calculation of the Amount of Glycine Neutralized by Sulfanilic Acid.—The total amount of glycine neutralized can be estimated by considering the expression

$$\overset{+}{\mathrm{H}} + \text{glycine} = \overset{+}{\mathrm{Gl}}\ \text{(glycine ion)} + \mathrm{HOH}$$

where

$$\frac{(\overset{+}{\text{Gl}})}{(\overset{+}{\text{H}})\ (\text{glycine})} = K$$

Reading the titration curve at various known ratios of glycine ion concentration to that of unneutralized glycine, K may be evaluated. For example, when the glycine is half neutralized, $K = 1/(\overset{+}{\text{H}}) = 223$, since 0.02 mol of glycine is seen to be neutralized at a pH of 2.35 or a cH of 0.0047. In a like manner for the following ratios the corresponding values of K are obtained.

Ratios.	pH	K
0.333	2.8	211.
0.500	2.64	218.
1.000	2.35	223.
2.000	2.05	224.
3.000	1.9	238.
Mean..		223.

From the value of K the amount of glycine neutralized at any pH can be calculated. In the presence of NaOH this value is, through the pH range included in Table V, also the amount of glycine neutralized by the sulfanilic acid. When HCl is also present one must subtract from the total glycine neutralized the amount neutralized by the HCl. This can be satisfactorily approximated by putting it equal to the sum of the increase in glycine ion and the decrease in sulfonate ion at any pH, referred to their respective concentrations in the absence of the HCl.

Table V gives the results of such calculations for the mixture represented in Fig. 1. Above the center line the mixture contains NaOH, while below, it contains HCl. Glycine ion is represented by $\overset{+}{\text{Gl}}$, sulfonate ion by $\overset{-}{\text{S}}$, and their respective tabulated increments by $\Delta\overset{+}{\text{Gl}}$ and $\Delta\overset{-}{\text{S}}$.

The mols of glycine neutralized by the sulfanilic acid are seen to pass through a maximum when there is neither HCl nor NaOH present. Such a mixture has a pH of 2.74. The theoretical value for the isoelectric point of such a system, it will be remembered from the first paper of this series, is obtained from the expression

$$(H)^+ = \sqrt{\frac{K_a\ (HAOH)}{K_b'\ (HBOH)}\ K_w}$$

where, in the present case, K_a is the acid ionization constant of the sulfanilic acid, 7 × 10^{-4}, and (HAOH) is its concentration in unionized form; and K_b' is the basic ionization constant of the glycine, 2.2 × 10^{-12}, and (HBOH) is its concentration in unionized form. Substituting the proper values, one is lead to the theoretical pH value for the isoelectric point of this mixture of 2.73 in place of the observed value of 2.74.

TABLE V.

Concentration of HCl or NaOH.	cH	$\overset{+}{\text{Gl}}$	$\overset{-}{\text{S}}$	$\Delta\overset{+}{\text{Gl}}$	$\Delta\overset{-}{\text{S}}$	Glycine neutralized by HCl.	Glycine neutralized by sulfanilic acid.
.0267	.00033	.00273	—	—	—	—	.00273
.0133	.00083	.0062	—	—	—	—	.0062
.0075	.00117	.00825	—	—	—	—	.0083
.0030	.00141	.00954	—	—	—	—	.0095
.00133	.00158	.01040	—	—	—	—	.01040
.0000	.00183	.01155	.0119	—	—	—	.01155
.001	.00186	.0117	.01175	.00015	.00015	.00030	.01140
.002	.00191	.0119	.0115	.00035	.0004	.00075	.01115
.003	.00207	.0126	.01085	.00105	.00105	.0021	.0105
.006	.00237	.0138	.0098	.00225	.0021	.00435	.00945
.009	.00266	.0149	.00895	.00335	.00295	.0063	.0086
.012	.00329	.0169	.00755	.00535	.00435	.0097	.0072
.018	.00439	.0197	.0059	.00815	.0060	.01415	.00555
.025	.0062	.0232	.00436	.01165	.00754	.01919	.0040
.040	.0124	.0294	.0023	.01785	.0096	.02745	.00195
.060	.0261	.0341	.0011	.02255	.0108	.03335	.00075

Other mol ratios yield analogous results. Table VI compares the observed pH of the isoelectric points of various mixtures of glycine and sulfanilic acid with the calculated values. These calculated theoretical values, it will be remembered, correspond to the pH at which the negative sulfonate ion concentration is equal to the positive glycine ion concentration. They are obtained by solving the following two simultaneous equations for y.

$$\frac{y \cdot x}{c - x} = K_a \quad \text{and} \quad \frac{K_w/y \cdot x}{c' - x} = K_b'$$

where y is the hydrogen ion concentration corresponding to the pH of the isoelectric point of the system;

x is the sulfonate (or glycine) ion concentration at this pH, which is the same in case of both ions;

c is the total sulfanilic acid concentration, and K_a its acid ionization constant;

c' is the total glycine concentration, and K_b' its basic ionization constant.

TABLE VI.

Concentration of sulfanilic acid.	Concentration of glycine.	pH observed.	pH calculated.
.043	.040	2.74	2.73
.01	.09	3.40	3.44
.03	.07	2.98	3.01
.04	.10	3.06	3.03
.05	.10	2.98	2.97
.10	.10	2.74	2.75
.02	.02	2.76	2.75
.10	.05	2.58	2.54
.08	.04	2.57	2.54
.07	.03	2.51	2.50

Table VII gives certain results of the same nature as those included in Table VI but for a few other pairs of substances. In connection with the work presented in the following paper a sample of lysine was prepared from hydrolyzed casein. The ionization constants of this substance are evidently not very accurately known, probably since it is difficult to prepare it with a high degree of purity. Scudder (7) gives for K_a about 1×10^{-11}, and for K_b "less than 1×10^{-7}." From Tague's titration of lysine dihydrochloride (8) one may calculate the value of K_a, which is found to be 1.2×10^{-11}, but, though the second basic ionization constant can be obtained from his curve, the first, which is the significant one, cannot. Solutions of the sample prepared for this work gave a pH to water of 8.8, which, using

1.2×10^{-11} for K_a, gives the value 5×10^{-8} for the first K_b. It is almost impossible to be sure that one has not a trace of sulfuric acid in such a preparation, however, and for the calculations of the theoretical pH values given in Table VII the value 7×10^{-8} was used. The agreement between the observed and calculated values of the isoelectric points of these systems, though still quite satisfactory, is not so good as in Table VI. This may be due partly to the problematical value of K_b, for lysine used in calculating the theoretical values, and partly to the fact that small changes in the mol ratio have a much larger effect on the pH than is true for glycine and sulfanilic acid, and thus small errors in the total concentration, which may be the case for the lysine, will contribute largely to the discrepancies. The agreement is, however, considered good.

TABLE VII.

	pH observed.	pH calculated.
0.04 sulfanilic : 0.02 lysin........................	3.09	3.15
0.02 " : 0.02 "	4.88	5.0
0.02 " : 0.04 "	6.69	6.82
0.02 glycine : 0.01 lysin........................	8.05	8.09

It will be noted that in the last instance the glycine is playing the rôle of the acid constituent of the mixture, as it is now at a pH above its isoelectric point.

SUMMARY.

Electrometric titrations of glycine, sulfanilic acid, and various mixtures of the two have been made. These mixtures are shown to give a curve which, between their respective isoelectric points, is different from that of either substance. These mixtures have a maximum buffering power at a pH which can be theoretically calculated, and which has the characteristics of an "isoelectric point of the system."

Other pairs of ampholytes are shown to act in an analogous manner.

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PHYSIOLOGICAL ONTOGENY.

A. CHICKEN EMBRYOS.

XII. THE METABOLISM AS A FUNCTION OF AGE.

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The rates of absorption, storage, and elimination of energy are perhaps the best indices that we possess of the vitality of a living organism. Since it has been frequently verified that: [A] absorbed energy = [S] stored energy + [E] eliminated energy; and since, by the chemical analyses of embryos at successive ages, the rate of storage has been ascertained (1), it remains only to obtain the catabolic activity of the embryo in terms of age. This has been done by the manometer method for the estimation of oxygen, and the present communication reports the results. The initial estimations of CO_2 production reported elsewhere (2) were admittedly subject to a number of unknown variables determinable with difficulty, such as variations in the concentration of CO_2 in the embryo and in the albumin and yolk, together with the contribution made by the carbonates dissolved from the shell. These values are functional to the carbon dioxide tension about the egg, and since, moreover, they cannot be estimated with great precision, a statistical analysis of the data from a very large number of eggs would be necessary. For these reasons we chose to measure metabolism by the oxygen consumption.

Method.

A Warburg manometer was used, attached to a special glass vessel to contain the hen's egg. The egg rested upon glass tips projecting from the walls to suspend it above the bottom which was layered with 5 cc. of a 1.0 N NaOH solution.

The volume of each apparatus, and thus its constant, was obtained by the method of Warburg (3). Brodie's fluid was used in the manometer. The volume occupied by the egg contents in the vessel, *i.e.* the whole egg minus the air sac, was assumed to be equal to the weight of the egg minus the weight of the shell (approximately 6 gm.). In general, eggs of the same size and shape were selected.

A control was made with each test; at first in the form of a fertile egg of 1 day incubation, but later, when this was found unnecessary, with a vessel empty except for the alkali. The experiments were done in a constant temperature room, eliminating thereby the use of a water bath.

A thermometer ground into each glass cover registered the temperature, which averaged approximately 39.0°C. The small fluctuations which did occur were not found to affect appreciably the results. Moreover, it was found that the large surface of alkali exposed provided for maximum absorption of CO_2 without the necessity of shaking. Shaking for a minute prior to reading the manometer made no difference in the result. Nor was it found, when thin rubber tubes in which cold water flowed were run along one side of the vessel to cool the wall over this area and thereby to initiate by convection a regular circulation, as in Barach's (4) recently constructed human oxygen chamber, that any acceleration of CO_2 absorption took place.

About ½ hour (the length of time being judged by the behavior of the control) was allowed for conditions to reach equilibrium. After this time readings were taken at varying intervals during periods of 2 to 6 hours until repetition of approximately similar results made one confident of their reliability. During the intervals between tests, the manometer and vessel were connected with an oxygen bag, so that the concentration of oxygen within the vessel remained always the same.

To obtain values for the rate of oxygen absorption per gm. of body weight the following figures are necessary, (1) the constant for the vessel (previously calculated); (2) the manometer readings, (3) the weight of the whole egg, and (4) the weight of the embryo.

One phenomenon was observed which we have not been able to explain. The embryo of an incubation age over 16 days, even when connected with the oxygen bag, did not survive in the apparatus over 12 hours. Their metabolism after 3 to 6 hours gradually fell. If the vessel was fully opened to the air for a few minutes the embryo would revive. Apparently it had nothing to do with a lack of oxygen, accumulation of CO_2, or changes in the humidity; neither was there an accumulation of ammonia.

TABLE I.

Metabolism of Chicken Embryos as a Function of Age.

1	2	3	4	5	6	7	8	9	10	11
Age.	No. of observations.	By experiment. O_2 per day per gm. wet weight.	Standard deviation.*	From curve. O_2 per day per gm. wet weight.	O_2 per day per gm. dry weight.	Solid oxidized per day per gm. dry weight.	Solid stored per day per gm. dry weight.	Absorption per day per gm. dry weight.	CO_2 per day per gm. wet weight.	Resp. quot.
days		*cc.*		*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	
6	8	50.0	2.1	50.0	896	0.444	0.665	1.11	29.9	0.60
7	5	38.4	2.3	43.0	735	0.364	0.584	0.95	29.6	0.69
8	3	36.7	1.5	39.0	628	0.311	0.510	0.82	29.3	0.75
9	10	39.2	1.3	36.5	562	0.278	0.478	0.75	29.0	0.79
10	7	32.8	1.2	35.0	500	0.248	0.465	0.71	28.5	0.81
11	7	33.9	1.2	34.0	442	0.219	0.465	0.68	28.0	0.82
12	9	33.9	1.2	33.2	378	0.187	0.465	0.65	27.0	0.81
13	5	32.4	0.7	32.5	322	0.159	0.465	0.62	25.7	0.79
14	5	30.3	0.6	31.7	259	0.128	0.447	0.57	24.0	0.76
15	7	34.3	1.3	30.5	209	0.103	0.395	0.50	22.0	0.72
16	11	29.0	1.4	28.7	175	0.087	0.320	0.41	20.1	0.70
17	5	26.1	1.0	26.2	152	0.075	0.250	0.33	18.1	0.69
18	6	21.1	1.3	23.2	131	0.065	0.215	0.28	16.2	0.70
19	3	20.5	0.3	20.0	113	0.056			14.2	0.71

Column 6 = figures calculated by the aid of values for the percentage of solid substance previously determined (Murray (1)).

Column 7 = values in Column 2 divided by 2019.3 (amount of oxygen absorbed when 1 gm. of fat is burned).

Column 8 = figures previously obtained (Murray (1), Table III).

Column 10 = figures read from smooth curve previously obtained (Murray (5)).

RESULTS.

The results of the oxygen determinations (Table I) may be seen (Fig. 1) to demonstrate a decrease in metabolic rate per gm. of body weight with age. This conclusion confirms that reached when the carbon dioxide was determined; except that in a more precise analysis and comparison of the results it appears that the oxygen estimations show a sharp fall of metabolism during the first days of the period under observation, whereas the carbon dioxide figures do not (5). As has been mentioned in the introductory remarks, however, numerous complications arising from the variability of unknown factors cast doubt upon the value of CO_2 elimination as a measure of catabolic change.

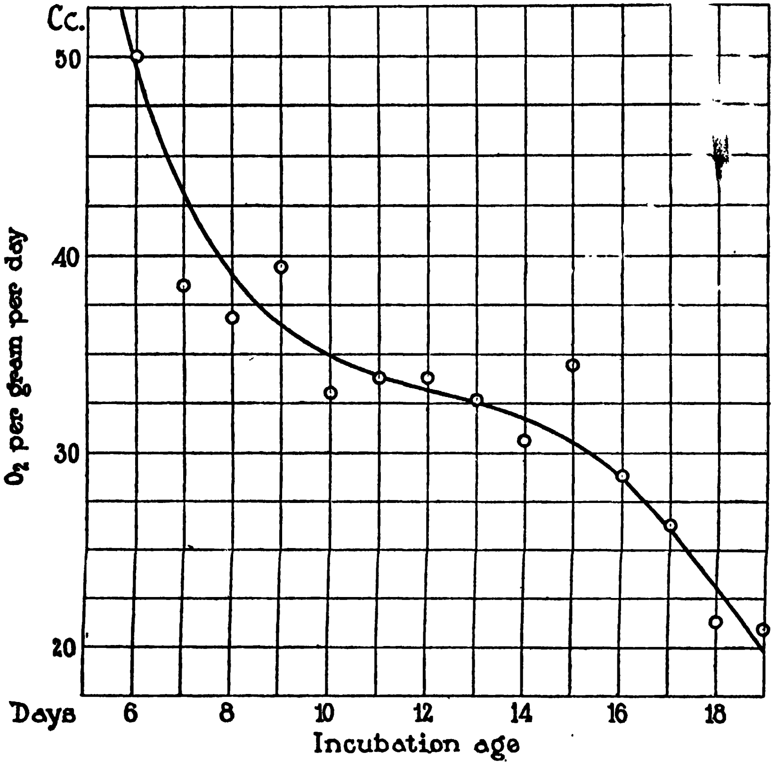

FIG. 1. Oxygen consumption in cc. per gm. of wet weight of chick embryo per day, as a function of age.

The oxygen determinations on the other hand were well controlled and presented no obvious factor to vitiate their use as indices of metabolic activity.

To oxidize 1 gm. of fat approximately 2000 cc. of O_2 are absorbed; whereas to oxidize 1 gm. of protein (966.3 cc.) or starch (828.8 cc.) about 900 cc. of O_2 are used (6). The total oxygen consumption for the first 19 days, estimated by graphical integration, comes to 2988 cc., which on the basis that only fat is burned during incubation leads to the conclusion that 1.48 gm. of dry substance (*i.e.* fat) is oxidized during that period. If only protein and starch were burned, it would require over 3.28 gm. to use the observed amount of oxygen. Previous chemical analyses have shown that approximately 1.62 gm. of substance is burned during the first 19 days, a figure which may now be accounted for on the assumption that 92 per cent of the metabolism is oxidation of fat, and the rest of protein and carbohydrate. This value is to be compared to 98 per cent fat oxidation found by measuring the CO_2 output. The former figure is probably more accurate.

During the last 5 days of incubation, when about four-fifths of the total oxidation takes place, the respiratory quotient is approximately 0.71, which points to fat consumption, during this period. The earlier values for the respiratory quotient are somewhat higher (up to 0.81); but they are variable and it is uncertain whether they deserve consideration. The results point to some error during the first 3 days when the CO_2 figures, and thus the quotient, also seem to be definitely too low.

If we discard the carbon dioxide estimations in favor of these later O_2 determinations and assume as we may without undue error that catabolism is at the expense of fat, we arrive at some notion of the changes in the metabolic rate with age.

Regarding the organism energetically and dynamically, the amount of energy exchange measures its activity or vitality. Hence, the amount of energy stored plus the amount set free might be used as a criterion of aliveness. By adding the rate of storage in terms of weight (previously obtained) to the rate of elimination, likewise in terms of weight as measured by oxygen usage, one obtains the desired value; namely, the rate of dry mass absorption per gm. of body weight per day (Table I). It may be seen (Fig. 2) that there is a marked fall with age in the rate of absorption expressed in these terms. Reasons have been enumerated for believing that during the first half of incubation, when the amount of metabolism is small relative to the total metabolism during incubation but large relative to the weight of the embryo, there is a not inappreciable amount of protein and carbohydrate oxidized. If this were a fact a straight line rather than an S-shaped curve as graphically represented might be indicated.

The consumption of food during the early days is enormous. On the sixth day for instance the embryo absorbs over its own mass of dry substance. Assuming that the water content of the diet is approximately that of the tissues, this would be equivalent to a mature man eating about 150 pounds of food per day. During the 12 days under observation, however, the percentage rate of absorption falls

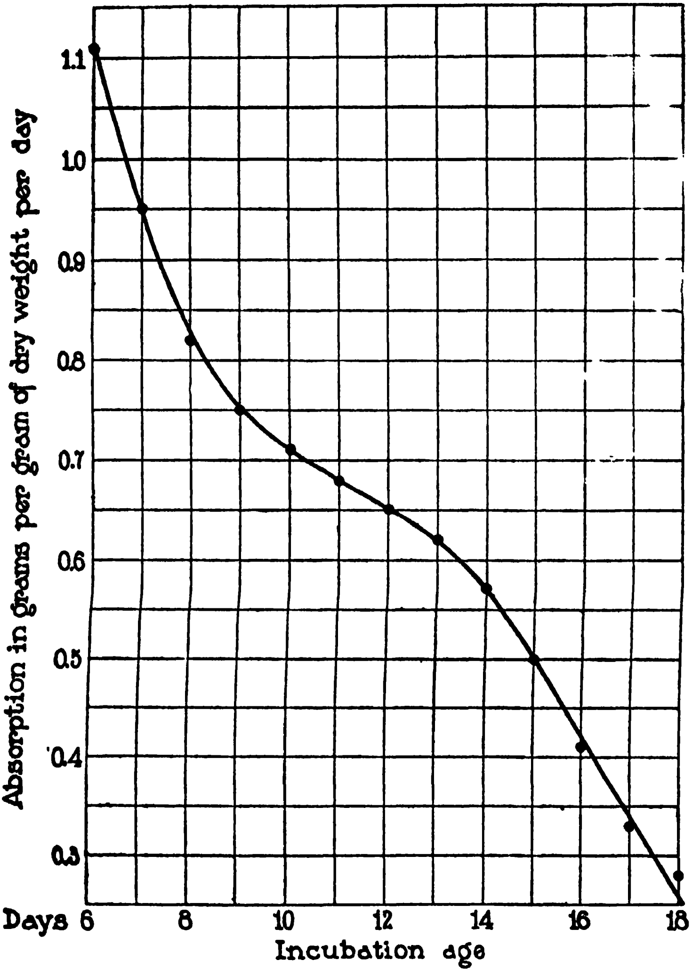

FIG. 2. Absorption of solid matter in gm. per gm. of dry weight of chick embryo per day, as a function of age.

to about 25 per cent (one-fourth its earlier value). According to Lotka, a mature meadowlark consumes about 6.6 per cent of its own weight a day (7) which would suggest a fall in absorption rate during the postembryonic period of a degree comparable to that which occurs during the 12 days before hatching.

SUMMARY.

1. The previous findings that the rate of metabolism per gm. of body weight decreased with age, and that during the incubation period catabolism was mostly at the expense of fat, have been confirmed.

2. These determinations of the rate of oxygen uptake have afforded more precise values for the catabolic rate and thus permit estimations of the changes with age in the rate of absorption.

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REGULATION OF THE HYDROGEN ION CONCENTRATION AND ITS RELATION TO METABOLISM AND RESPIRATION IN THE STARFISH.

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The composition and condition of the body fluids of animals show departures from those of their environment usually corresponding to their degree of development. In the cœlenterates and sponges a free circulation of sea water accomplishes the distribution of materials. The interior and exterior are then exposed to practically the same medium. In the starfish, however, there are a closed cœlomic cavity and a digestive system which are not swept by a constantly renewed supply of sea water. The fluids contained in these spaces are similar to sea water, but modified by the enclosing tissue so as to be true components of the organism. The hydrogen ion concentration of the body fluids becomes progressively greater in the deeper regions of the organism, establishing a gradient from the interior toward the exterior. The normal reactions of these fluids are shown here to be optimal for several representative metabolic processes.

In the starfish there is a mouth-opening situated on the lower side, through which the soft cardiac portion of the stomach is extruded to surround food. In this stomach digestion proceeds until the food is sufficiently disintegrated to permit its withdrawal into the body. The partially digested food is then swept by ciliary currents through the pyloric cæca, where digestion and absorption are effected. Then, apparently, the products of digestion pass through the wall of the cæcum into the surrounding cœlomic fluid. Here again ciliary currents, together with body movements, serve for distribution (Irving, 1924–25).

The investigations presented were carried out with two forms of starfish common in the vicinity of the Hopkins Marine Station at Pacific Grove, California. These forms were *Pisaster ochraceus* and *Patiria miniata*. *Patiria* is short-armed, thin-walled, and much more tractable in aquaria. It was therefore used for most of the experiments, although many of them were also tried on *Pisaster* with corresponding results.

Normal pH of Stomach, Cæca, and Cœlomic Fluid.

The following preliminary experiments showed that the stomach is distinctly more acid than sea water. Mussel meat stained with neutral red takes the deep red color of a pH less than 7. During cardiac digestion of such meat, the red color is retained, and the stomach walls take and keep for several days a pink color indicating a hydrogen ion concentration near neutrality. Several times I was able to pipette enough fluid from the stomach through the mouth to show a pH between 7.3 and 7.5 with phenol red. This fluid is already much more acid than the sea water with which it is in such relatively close contact.

After ingestion of stained mussel meat the oral lumina of the cæca stain deep red with neutral red, the remaining regions being naturally too dark to show the dye. The red color remains conspicuous in the lumen for a week, during which time this particular cæcum region plainly remains more acid than sea water, but not far from neutrality. Although the neutral red color in the dark cæca is difficult to compare directly, the stained cæcum changes color distinctly when placed in a more alkaline or acid buffer solution. A series of cæca stained by ingestion of neutral red were placed in buffer solutions, and, from observation of the solution producing the least change, the cæcum pH was estimated to lie between 6.6 and 7.0. This is quite contrary to the observations of Roaf (1909–10[1]) who declares that the cæca of *Asterias rubens* become alkaline after digestion is completed.

Samples of cœlomic fluid, which surrounds the cæca in the body cavity, showed the following acidities:

Patiria	pH	8.0	7.7	7.6	7.6	7.6	7.6
Pisaster......	pH	7 5	7 6	7.5	8.1	7.6	7 6
Sea water	pH	8.3					

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[1] Roaf (1909–10), p. 448.

These are typical of many determinations taken at various periods before and after feeding, and, considering the difficulties from mixture with sea water and a slight opalescence, indicate a consistent normal close to pH 7.6. McClendon (1916–17) found the pH of cœlomic fluid of the sea urchin *Toxopneustes variegatus* between 7.7 and 7.8, while Crozier (1918) found the cœlomic fluid of the holothurian *Stichopus mœbii* at 7.6. Cœlomic fluid is also different from sea water in its salts. The freezing point depression of a sample from *Patiria* was determined by Dr. J. P. Baumberger to be 1.885, compared with 2.075 for sea water.

TABLE I.

Changes in the Hydrogen Ion Concentration of Sea Water Caused by Excised Cæca.

Experiment No	0 hrs.		20 hrs. Cilia condition.		45 hrs. Cilia condition.	Comparative activity.
	pH	*pH*		*pH*		
1	5 0	6 0	Dead.	5 8		0
2	5 6	6 3	o.s. active. i.s. dead.	5 8	o.s. slightly active. Digestion started.	1
3	6 0	6 35	o.s. active. i.s. active.	6 0	o.s. slightly active. i.s. dead.	2
4	6 2	6.35		6 2	o.s. active. i.s. dead.	3
5	6 4	6 4		6 2	o.s. active. i.s. slightly active.	4
6	6 6	6 35		6 2	o s. active. i.s. slightly active.	3 5
7	6 8	6 55	o.s. active. i.s. active.	6 2	Dead.	0
8	7 0	6.85		6 7	o.s. slightly active. i.s. dead.	1
9	7.4	6.95	o.s. active. i.s. active.	6 8	o.s. active. i.s. active.	4
10	7 8	6 9		6 7	o.s. active. i.s. dead.	1
11	8 4	6 9	o.s. active. i.s. active.	6 4	o.s. active. i.s. dead.	1

o.s. = outside, toward cœlom; i.s. = inside.

These observations show that the fluids and organs of *Patiria* and *Pisaster* are maintained at an acidity quite different from that of sea water.

The Optimum for Survival.

After the discovery that the internal organs and fluids have each a normal, regular pH, they were next subjected to environments varying in this respect. In order to start under reproducible conditions, sea water acidified with HCl was aerated until a constant pH was reached, indicating the arrival of the solution at carbon dioxide equilibrium with the air.

TABLE II.

Changes in the Hydrogen Ion Concentration of Sea Water Produced by Excised Cæca

Experiment No	*Start*	17 hrs	21 hrs	45 hrs
	pH	*pH*	*pH*	*pH*
1	5 0	6 2	6 1	6 0
2	5 4	6 3	6 3	6 1
3	5 8	6 3	6 3	6 1
4	6 0	6 7	6 7	6 2
5	6 2	6 3	6 3	6 1
6	6 4	6 3	6 3	6 2
7	6 6	6 3	6 3	6 1
8	6 8	6 7	6 7	6 7
9	7 0	6 8	6 7	
10	7 2	6 8	6 7	
11	7 4	6 8	6 7	6 6
12	7 6	6 8	6 8	6 6
13	7 8	6 8	6 8	
14	8 0	6 6	6 5	
15	8 2	6 7	6 7	
16	8 4	6 7	6 7	

To such solutions in test-tubes, excised cæca were added, one in each tube. Table I shows the changes which characteristically occurred in these experiments, and Table II extends the results. The condition of the tissues was determined in each case by examination with a binocular dissecting microscope.

These experiments show a tendency of the excised, living cæca to alter the medium toward a pH of about 6.7. When the pH fell below 6.7 to about 6.3, disintegration and injury to the cilia were apparent. The halt at pH 6.7 is too distinct to represent gradual decomposition, and the subsequent rapid fall to pH about 6.3 indicates a change to a new status. Probably the lower pH, which is concomitant in appearance with physical signs of tissue death, represents the stage of autolysis. It is apparent that the living animal maintains a rather definite normal reaction of its cæca, and that the cæca themselves when excised are capable of producing the normal reaction in sea water media initially quite different.

All of these tests dealt with living tissue, except as indicated. It was not a matter of autolysis or putrefaction because of the persistence of ciliary action and the absence of the conspicuous odor of dead marine animals. These soft tissues disintegrate and produce unpleasant odors almost immediately after ciliary action ceases.

The Optimum pH for Digestion.

Many of the older physiologists investigated the problem of digestion in echinoderms and produced conclusions of quite contradictory nature. Abderhalden (1911[2]) lists seven different enzymes named in echinoderms by various workers. The names appear, however, to be usually assigned by analogy with mammalian enzymes on the basis of substance digested and conditions of action, quite apart from normal conditions. Beyond these analogies, based often on rather indefinitely reported observations, there is little precise evidence of the nature of the enzymes. It is clear that the cæca produce digestive enzymes (Frédericq, 1878; Cohnheim, 1901; van der Heyde, 1922, 1923). These enzymes easily bring about autolysis of the organs, but extracts behave in an extremely slow fashion. Furthermore, the technique applied to their study has not been of the sort calculated to give quantitative results.

Frédericq (1878) first clearly showed that proteolytic digestion by the cæca occurred near the neutral reaction. Other invertebrates probably carry on digestion naturally near neutrality, and not in the strongly acid condition where pepsin finds its optimum. Darwin's observations on digestion in the earthworm show a normal reaction near neutrality (Darwin, 1890). Bodansky and Rose (1922[3]) extracted a digesting substance from jelly fish which had one optimum for gelatin liquefaction at pH 2.6, and another, much more favorable, at pH 7.3. The optimum at pH 2.6 seems too far removed from the normal reaction of sea water, which circulates so freely through the animal, to be important under vital conditions. The empty stomach of *Stichopus* was found by Crozier (1918) to have a pH between 5.0 and 6.5, while during feeding it ranged from 4.8 to 5.5. Crozier remarks that this animal's calcareous diet may constitute a special condition which is met by the relatively strongly acid digestive juices. Roaf's observations of cH 10^{-4} during digestion in *Asterias rubens*[1] suggest an acidity far greater than any which I observed in *Pisaster* or *Patiria*, although they are similar forms. Van der Heyde (1922) observed in the stomach of *Asterias* pH 7.1, 7.6, and 7.7, and in the cæca 7.3. The chemical methods and biological conditions involved in determining the normal hydrogen ion concentration during digestion must be rather precisely established, so that many casual observations require critical examination. From the available evidence and the biological conditions a natural reaction far from neutrality would be unexpected.

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[2] Abderhalden (1911), p. 538.

[3] Bodansky and Rose (1922), p. 475.

Because of the difficulties found in separating active extracts, digestions by excised surviving cæca *in vitro* were used. These are here nicely applicable because of the appropriateness of the cæca and the excellent criterion of their survival in ciliary activity.

Pipettes were prepared with their tips protected by rubber tubing, the opposite end bearing a short length of rubber tubing and a pinch-cock. 2 cc. of the solution to be digested were introduced into the pipette, its lower end stopped, and the pinch-cock closed. By slightly withdrawing the closed rubber tube from the pipette the solution could be retained without loss. Next the rubber-protected end of the pipette was inserted into the open (pyloric) end of a previously weighed cæcum and the cæcum tied on with silk. The cæcum was then drained and transferred to a test-tube containing 10 cc. of sea water prepared at constant pH. The pipette and cæcum were suspended in the solution by a cork, the pinch-cock opened, and the solution allowed to run into the cæcum. The cæcum became distended and remained distended, if uninjured, for 2 days or more. In this way a sack of living digestive tissue was produced, with a capacity in the case of large cæca of over 3 cc. Products of protein digestion diffused through the cæcum wall and were determined by the Van Slyke method for amino nitrogen.

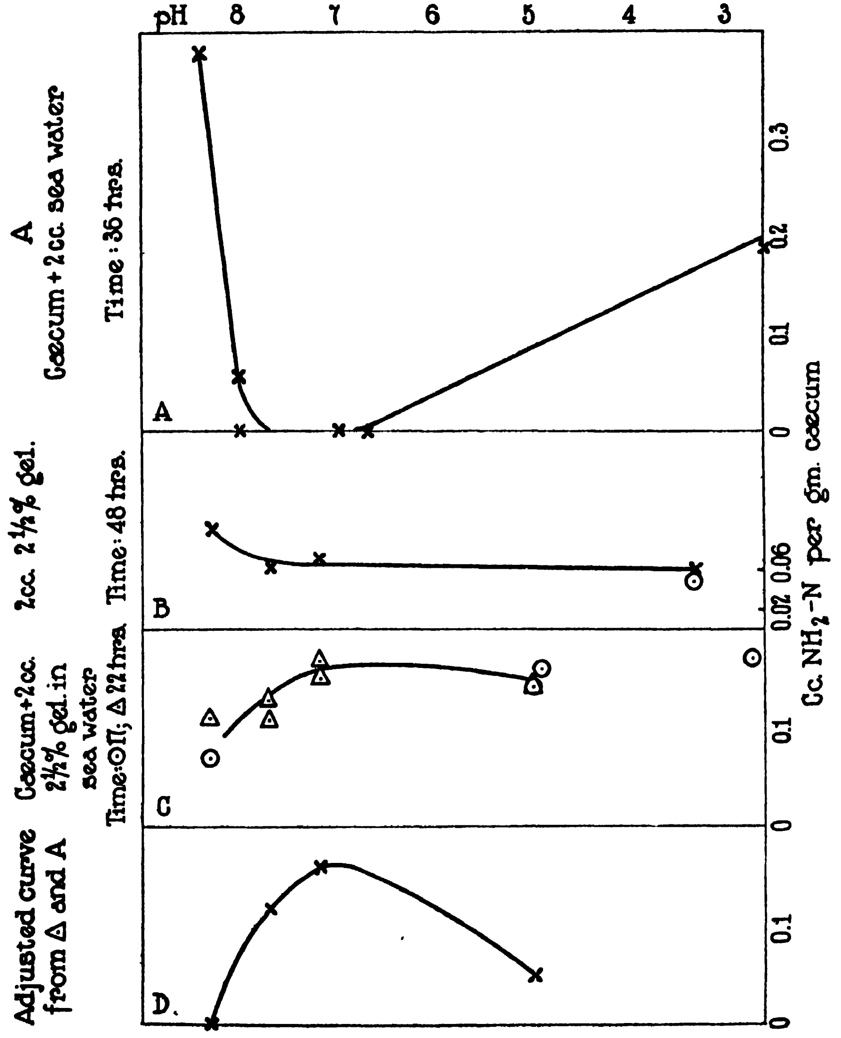

FIG. 1.

An appreciable production of amino nitrogen might be expected from the cæca themselves. In order to determine the normal amino nitrogen production, 2 cc. of sea water acidified and equilibrated by aeration were introduced through the pipette into the cæcum. The cæcum was then immersed in 10 cc. of the same solution. After an interval of time a 2 cc. sample of the external liquid was removed and its amino nitrogen determined. The new pH of the solution was also noted. In this way the amino nitrogen production of the cæcum alone could be determined. Data for the results are indicated in Fig. 1, Curve A.

These control experiments indicate a minimum amino nitrogen production at about the neutral point, increasing on each side. The neutral reaction has already been shown to be near the normal for cæca, and consequently tissue death and self-digestion would be expected to be less. Bacterial action is not excluded, but if present is least effective in producing amino nitrogen at neutrality. Evidently also the energy consumed by the tissue during survival is not produced from protein, or the amino nitrogen would increase at the point of greatest activity.

To determine the effect on food digestion of various hydrogen ion concentrations, a 2½ per cent solution of gelatin in sea water was used. Control determinations of the amino nitrogen production of this stock solution during 48 hours are shown in Fig. 1, Curve B. 2 cc. of the solution were added to 10 cc. of the sea water used in the experiments. The time of these controls is double that in the experiments on gelatin digestion, and yet the amino nitrogen production is very small.

The digestive effect of cæca on gelatin was tried in solutions at various hydrogen ion concentrations. All sea water used was acidified with hydrochloric acid and then aerated to constant pH. At the time of digestion determination, pH was again determined and the results were found in accord with previous experiments. Results are indicated by Curve C in Fig. 1, where two typical experiments are recorded together. The curve presents directly the appearance of an optimum for digestion near pH 7. The greater amino nitrogen production in acid reactions is obviously, from Curve A, attributable to autolysis. When a curve is adjusted by deduction of amino nitrogen production as in Curve A from that in Curve C, the resulting curve, D, plainly shows an optimum near pH 7.

After these indications that the optimum digestion conditions were also those of the normal organism, attempts were made to prepare a digestive extract whose activity might be compared at different acidities. Various extracts were prepared by permitting cæca to autolyze with alcohol or toluol. Products of such procedure caused only slow digestion, and the results did not show that any appreciable separation of the enzyme had been effected. As another method, many cæca were macerated with sand, spread on glass plates, and dried under low pressure at 40 degrees. The dried mass was then ground, but could not be redissolved or uniformly suspended on account of the fat present. Using this material in temporary suspension, the neutral reaction was obviously most favorable for the liquefaction of gelatin.

TABLE III.

Changes in pH of Sea Water Produced by Cæca during Different Time Intervals.

	Experiment No.	Weight of cæca.	Start.	Period 1.		Period 2.		Period 3.	
		gm.	*pH*	*hrs.*	*pH*	*hrs.*	*pH*	*hrs.*	*pH*
Aerated.	1	3.64	8.2	3.08	7.8	9.40	7.7	3.16	7.8
	2	4.37	8.0	3 00	7.5	9.40	7.4	3.16	7.3
	3	3.53	7.8	2.92	7.4	9.40	7.3	3.16	7.3
	4	5.34	7.3	2.84	7.1	9.40	7.3	3.16	7.2
	5	4.53	5.0	2.80	6.8	9.40	6.8	3.16	6.8
	6	4.53	3.8	2.70	6.1	9.40	6.4	3.16	6.5
Sealed.	7	6.33	8.1	2.92	7.9	3.25	7.5	20.0	6.8
	8	6.58	7.9	2.75	7.7	3.25	7.3	20.0	6.7
	9	6.13	7.7	2.66	7.4	3.25	7.1	20.0	6.7
	10	6.41	7.3	2.56	7.1	3.25	6.8	20.0	6.6
	11	6.42	4.9	2.25	6.2	3.25	6.5	20.0	6.4
	12	6.25	3.8	2.16	4.8	3.25	5.2	20.0	5.6

The Optimum pH for CO_2 Production.

Having shown that the optimum reaction for ciliary survival and digestion corresponds with the normal tissue reaction of about pH 6.7, it would be expected that this reaction would be most satisfactory for metabolism in general. Respiration, measured by CO_2 production, is the indicator used here for metabolic activity.

For the experiments uniform amounts of weighed cæca were placed in Pyrex flasks with sea water of known pH in equilibrium with the air. One series was aerated, the other sealed with rubber stoppers to retain CO_2 as produced by the cæca. Changes in the sea water of the aerated series should be then attributed to non-volatile substances or to a buffer effect of the tissues themselves; changes in the sealed series would include these and the change from CO_2. During the experiments none of the tissues putrified, and the times were all less than had been previously shown quite compatible with survival.

TABLE IV.

Changes in pH of Sea Water Produced by Cæca during Different Time Intervals.

	Experiment No.	Weight of cæcum.	Start.	Period 1.		Period 2.		Period 3.		Period 4.	
		gm.	*pH*	*hrs.*	*pH*	*hrs.*	*pH*	*hrs.*	*pH*	*hrs.*	*pH*
Sealed.	1	1.80	8.6	5.59	7.7	1.75	7.6	2.25	7.6	12.9	7.1
	2	1.46	8.5	5.40	8.0	1.75	7.8	2.40	7.6	12.9	7.1
	3	1.62	8.5	5.25	7.6	1.75	7.3	2.40	7.1	13.0	6.8
	4	1.34	8.2	5.08	7.1	1.59	6.8	2.40	6.7	13.0	6.7
	5	1.38	5.0	5.00	6.4	1.50	6.5	2.40	6.5	13.0	6.4
	6	1.28	4.8	4.75	5.0	1.50	5.8	2.40	6.1	13.0	6.2
Aerated.	7	1.88	8.6	5.92	8.3	1.42	8.3	2.75	8.3	12.4	7.8
	8	1.82	8.5	5.75	8.3	1.42	8.3	2.75	8.3	12.6	8.2
	9	1.61	8.5	5.59	8.2	1.42	8.2	2.75	8.1	12.6	7.8
	10	1.36	8.2	5.25	7.7	1.42	7.7	2.75	7.3	12.6	7.2
	11	1.38	5.0	5.00	7.2	1.42	7.3	2.75	7.0	12.6	6.8
	12	1.37	4.8	4.84	6.3	1.42	6.3	2.75	6.2	12.6	6.4

The results of these experiments, recorded in Tables III and IV, show in both the sealed and aerated series the previously observed progressive acidification of the solutions above pH 6.7 toward this reaction. In the aerated series the change is relatively slight after the first period, an effect which probably originates principally in neutralization by tissue substance.

In preparing acidified sea water solutions the excess base, and consequently buffer effect, were changed by addition of strong acid. Therefore only those experiments are comparable which were carried out with the same solution. These are recorded in the tables in the same horizontal line. In such solutions of similar excess base, hydrogen ion concentration change is directly proportional to carbon dioxide change in the range considered (McClendon, Gault, and Mulholland, 1917). The progress of these changes in the sealed series is indicated in Figs. 2 and 3, where vertical height represents change in hydrogen ion concentration per gm. of cæcum substance per hour.

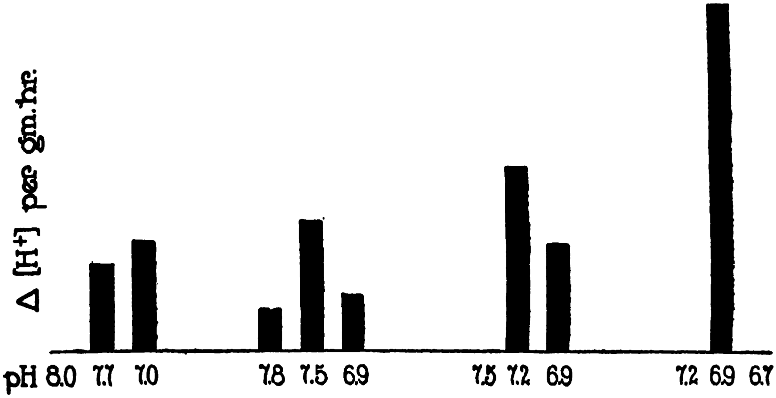

Fig. 2 (from Table III). $\Delta[H^+]$ per gm. per hour in sealed series, corrected by subtraction of $\Delta[H^+]$ of aerated series.

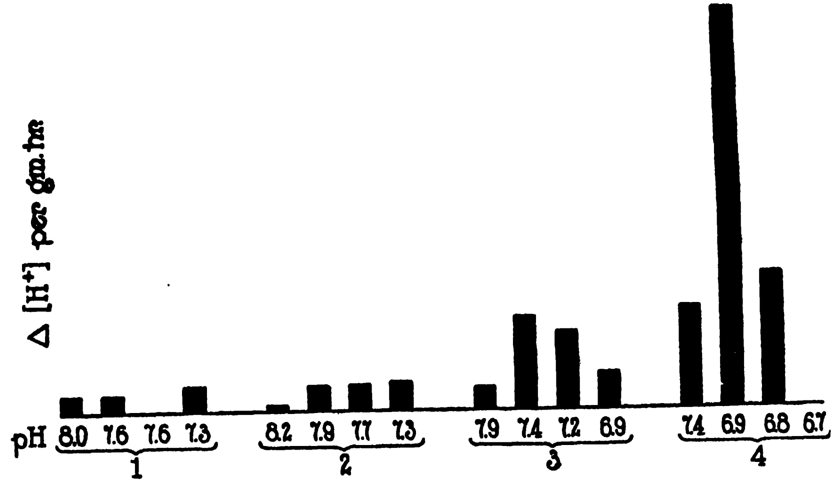

Fig. 3 (from Table IV). $\Delta[H^+]$ per gm. per hour in sealed series, corrected by subtraction of $\Delta[H^+]$ of aerated series.

These are corrected by subtraction of the change in the corresponding aerated experiment.

The figures show in each experiment a small carbon dioxide production in the first period, followed by a much greater production in the second period, and usually by diminishing changes in subsequent periods. That respiration during the first period was so small is remarkable. It is apparent that the cæcum fits its medium better after some time for adjustment.

The reaction about pH 7, close above that found normal for the cæca, seems most favorable to carbon dioxide production. It is not possible, however, to judge this point; for the diminished excess base of the initially more strongly acidified series causes a correspondingly decreased buffer value.

The experiments show that carbon dioxide is the principal acid serving to bring the cæca to the normal reaction. The aerated series also changes slightly toward the normal reaction, but this change occurred almost entirely in the first period and may be attributed to the buffer effect of the cæcum substance. No evidence appears to suggest the production of an appreciable amount of non-volatile acid.

Conditions of acidity represented by pH less than 6.7 have already been shown to be unfavorable to the cæca. Where the reaction fell below pH 6.7 a sharp drop was noticed to a rather stable reaction about pH 6.3, which attended death and decomposition. In the aerated series at pH 5.0 reported in the tables the cæca were able to recover the normal reaction of pH 6.8, while in the corresponding sealed series recovery was impossible. Evidently here the first harmful attack is on respiratory activity, which is unable to eliminate carbon dioxide into acid solutions of such feeble buffer capacity without aeration. If the removal of carbon dioxide is facilitated by aeration, respiration is possible and the tissues can overcome the unfavorable conditions by neutralizing the acid.

For living starfish it was shown that the normal reaction in the cæca was about pH 6.7, for cœlomic fluid which surrounds them, pH 7.6, and for sea water pH 8.3. To change sea water from pH 8.3–6.7 would require more than a fifty times increase in carbon dioxide tension (Henderson and Cohn, 1916). On the basis of this comparison the starfish evidently has quite a favorable pressure gradient for carbon dioxide elimination. The optimum might then follow only in consequence of the necessity of maintaining a carbon dioxide tension necessary to overcome the resistance to elimination. But each solution was initially in carbon dioxide equilibrium with the air, and all were altered by the cæca to or well on the way toward pH 6.7. In solutions of different buffer value this required carbon dioxide tensions of quite different magnitude, so that the optimum was sought irrespective of the carbon dioxide tension it entailed. The optimum is therefore concluded to apply to processes of metabolism in general, and not to the mechanical elimination of carbon dioxide alone.

The optimum pH found is not, then, one merely favorable to respiration by the establishment of a large pressure gradient. It is an optimum, furthermore, which applies to metabolism in general. It is well known that tissues usually have optimal hydrogen ion concentrations for their most conspicuous reactions; but it did not necessarily follow that all of the metabolic processes should coincide in an optimum for the tissue as a whole, at which each individual process is most effective.

CONCLUSIONS.

The cæca maintain for themselves a hydrogen ion concentration of pH 6.7, which is different from their immediate environment, the cœlomic fluid. This in turn differs from sea water. The capacity for maintaining these differences approximately constant requires an efficient regulatory system. This does not infer the existence of a special organ or physical system in the organism, but a fact in the operation of its metabolism. Regulation of hydrogen ion concentration in living mammalian blood has proved to be an essential fact in the existence of the organism, although we cannot explain its entire operation or significance. A regulation similar in effect has been generally assumed to exist in all organisms, although it is seldom demonstrated. It is hard to conceive vital substances which could be effective and still incapable of the adjustment and preservation of their own reaction within a comparatively restricted range. It is probable that ability to regulate and maintain a constant hydrogen ion concentration agrees in general with the degree of development of the organism as a whole.

SUMMARY.

The normal reaction of the cœlomic fluid in *Patiria miniata* and *Asterias ochraceus* is pH 7.6, and of the cæca, 6.7, compared with sea water at 8.3, all without salt error correction. A medium at pH 6.7–7.0 is optimum for the cæca for ciliary survival and digestion of protein, and is maintained by carbon dioxide production.' The optimum pH found for carbon dioxide production is a true one for the effect of hydrogen ion concentration on the tissue. It does not represent an elimination gradient for carbon dioxide.

Because the normal excised cæca maintain a definite hydrogen ion concentration and change their internal environment toward that as an optimum during life, there exists a regulatory process which is an important vital function.

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STUDIES ON ENZYME ACTION.

XXXIX. LIPASE ACTIONS OF EXTRACTS OF THE WHOLE MOUSE AT DIFFERENT AGES.

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INTRODUCTION.

The lipase or ester-hydrolyzing actions of extracts of the whole rat at ages from 3 days before birth until 3 years 15 days were presented in a previous communication.[1] The "pictures" of relative actions on ten esters were shown to change from a type similar to that found for the Flexner-Jobling rat carcinoma for the youngest rats to a type which may be taken to be characteristic of the adult rat and reverting to some extent for the oldest rats to the embryonic type. The absolute enzyme actions were also recorded and shown to undergo characteristic changes with increasing age of the rat.

A comparison of the ester-hydrolyzing actions of extracts of the whole rat and rat tumors with similar actions of whole mouse and mouse tumors presents a definite problem which it is the aim of this paper to answer. Rat embryos and the youngest rats showed actions similar to several rat tumors. Similar actions were found for a number of tissues of rabbit embryos.[2] Certain mouse tumors showed actions strikingly different from those of rat tumors.[3] The question which was raised was whether mouse embryos and the youngest mice would show enzyme actions similar to mouse tumors or to rat embryos and certain rat tumors.

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[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925–26, viii, 75.

[2] Noyes, H. M., Falk, K. G., and Baumann, E. J., *J. Gen. Physiol.*, 1925–26, ix, 651.

[3] Sugiura, K., Noyes, H. M., and Falk, K. G., *J. Cancer Research*, 1925, ix, 129.

Experimental Methods.

Brown, and in some cases, black mice, male and female, were used. They were obtained from the stock of the Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, Long Island, through Dr. C. B. Davenport and Miss Dorothy Newman, who made possible the satisfactory carrying out of this investigation. The mice which were used were normal as far as could be told. They were killed with ether, passed through a meat chopper twice, and water and toluene added at once. The extracts were made up with water to a volume of 25 cc. per gm. of ground material. After standing overnight, the mixtures were filtered through paper, cloudy or turbid liquids being obtained. These were brought to pH 7.0, diluted 50 per cent with water, and used for the enzyme tests. The conditions of testing the lipase actions were the same as those described previously; 15 cc. of solution, 3.4 milli-equivalents of each of the ten esters, 22 hours incubation at 37–38°, titration with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator, duplicate and blank determinations, toluene present throughout. The concentrations of the mixtures tested corresponded to 26.7 mg. of original material per cc. of mixture tested.

EXPERIMENTAL RESULTS.

The experimental results will be given only for mice whose ages were definitely known. The following data include the book numbers of the experiments (for reference); the ages of the mice in days, negative values referring to number of days before birth, zero ages to the fact that the mice were killed immediately after birth; and the weights of the mice in gm., or in the experiments with very young mice where more than one was used, the average weights. The ages of those which were obtained before birth were calculated on the assumption of a gestation period of 21 days.

Book No.					5B	12B	13B	2	3	4	17
Age of mouse, *days*					−6	−6	−1	0	0	4	5
Weight " " *gm*					0.24	0.52	1.30	1.51	1.60	2.47	2.50
Book No.	6	16	9B	9A	7	36	18	8A	8B	33	19
Age of mouse, *days*	7	8	9	9	12	15	17	20	20	23	27
Weight " " *gm*	3.50	4.63	4.36	4.70	6.00	6.29	4.78	4.63	5.50	7.46	8.50
Book No.	41	22	24	27A	27B	25	28	39	40	47	48
Age of mouse, *days*	29	39	39	40	40	42	46	46	46	46	47
Weight " " *gm*	9.64	14.2	12.0	8.68	10.1	12.9	13.7	17.6	7.87	17.3	18.3
Book No.	49	23	29	50	42	30	10A	10B	51	34	35
Age of mouse, *days*	48	48	48	49	50	52	53	53	54	55	59
Weight " " *gm*	16.7	14.6	10.4	20.6	13.0	11.8	12.0	12.3	20.3	12.2	16.0
Book No.	38	32	20	1B	1A	45	26	31	44	46	11
Age of mouse, *days*	71	89	113	161	161	185	191	241	246	289	333
Weight " " *gm*	14.8	18.6	17.9	21.6	20.8	24.0	19.1	31.3	25.3	32.8	29.6
Book No.	15	43	21	37	52						
Age of mouse, *days*	437	452	466	557	626						
Weight " " *gm*	32.3	38.1	26.8	31.1	21.9						

The weights of the mice did not increase regularly with their age. Occasionally the weights differed considerably even with mice of the same age and litter. The 46 day old mouse, No. 40, was about half the weight of three other mice of the same age, due, however, to a malformation of the jaw and teeth which evidently interfered with its food intake. Autopsy showed no other abnormality.

The relative actions on the esters are shown in Fig. 1. Three ages are shown in each of the first three charts, four in each of the remaining nine. Where two or more series were studied at the same age, and the results did not differ appreciably, these are averaged and presented as one series. Where marked differences were observed for two series at the same age, both curves are given. Each curve represents a set of results at a definite age. The order of arrangement of the esters is the same as in previous papers, and, in general, a similar method of treatment of the results is employed.

A study of the curves in Fig. 1 brings out the fact that there is a difference in the "pictures" of the youngest mice in comparison with the

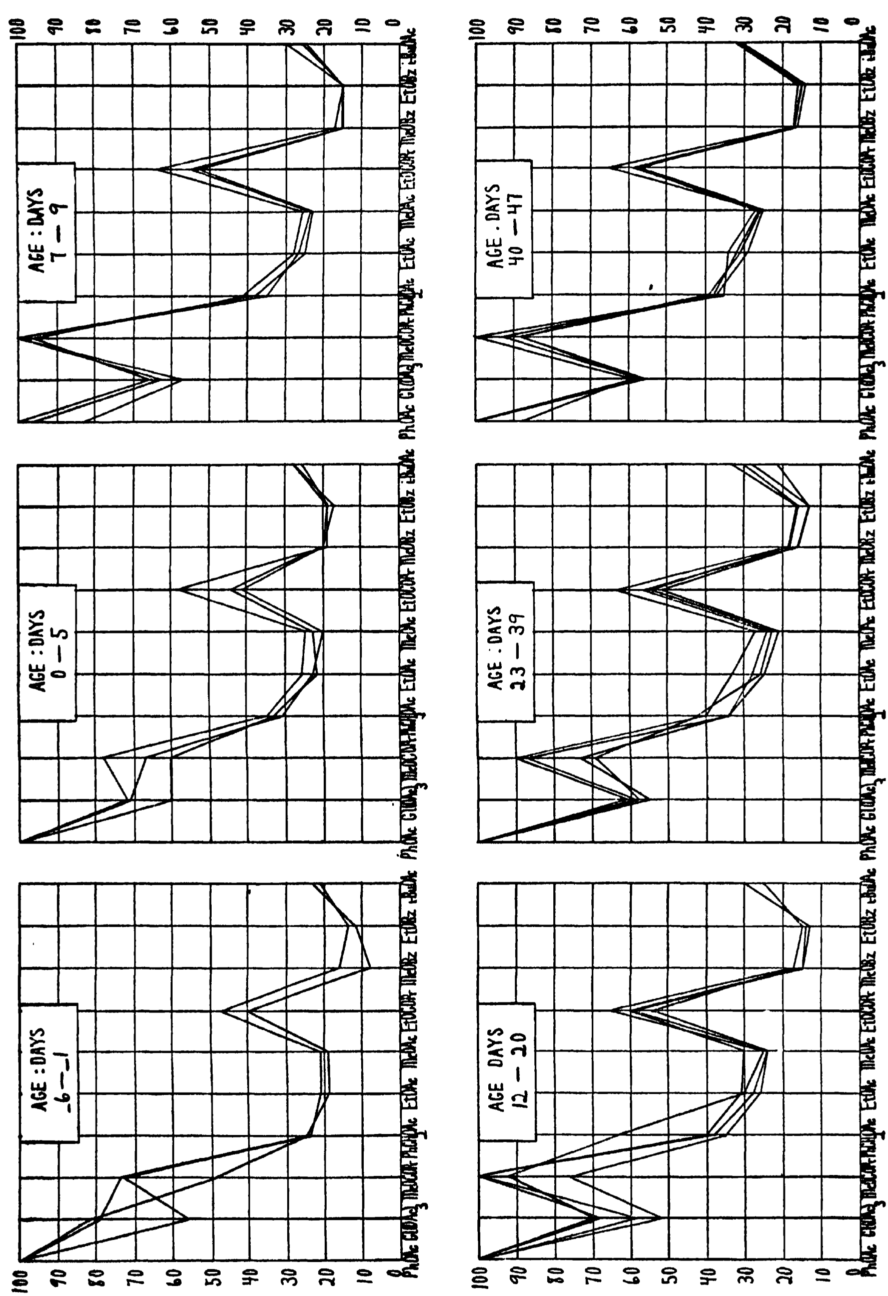
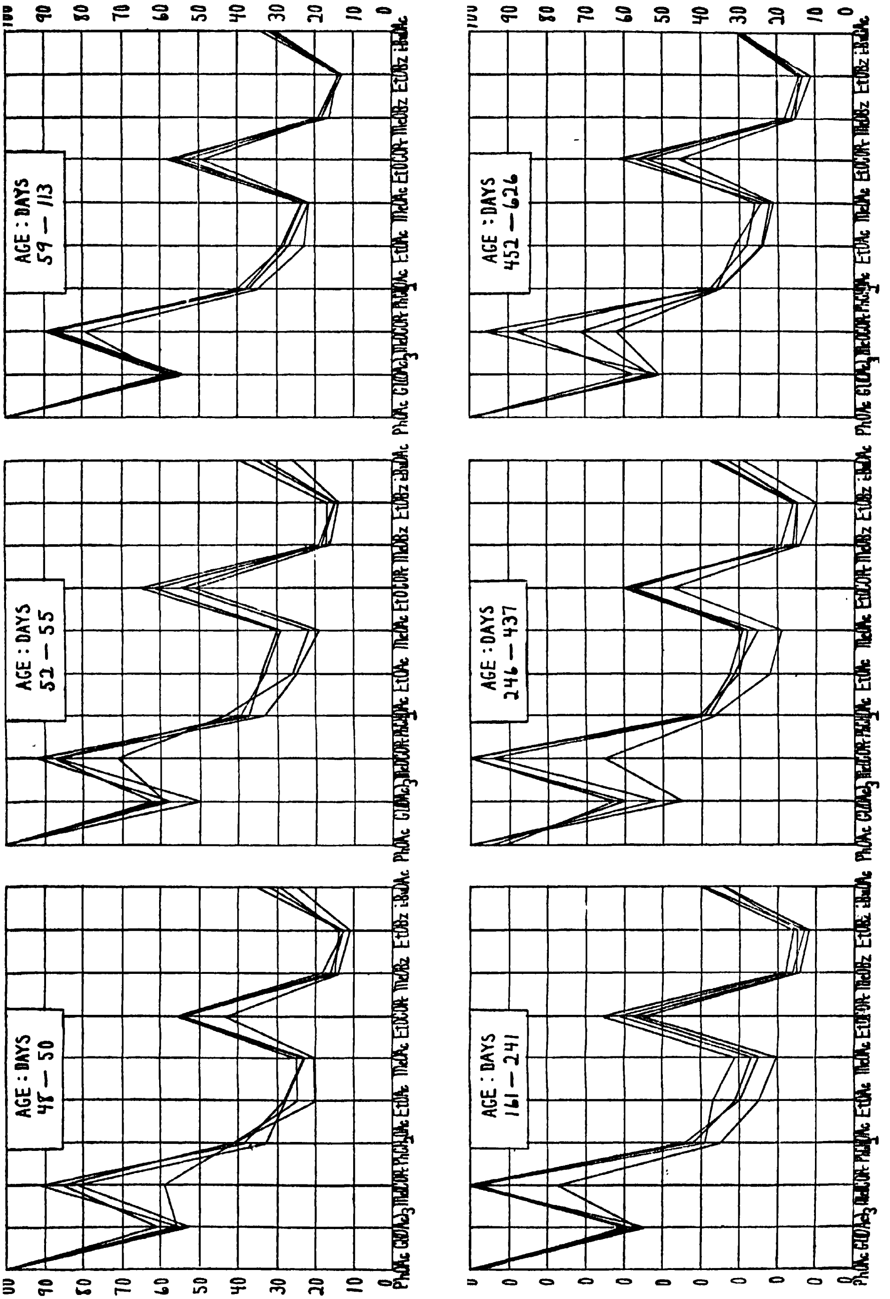


FIG. 1. Whole mice of different ages. Relative lipase actions, 45 curves, grouped in 12 plots, each curve representing the relative hydrolyzing actions of the extract of a mouse (or several mice) of definite age on the esters indicated on the abscissa axis. The ages of the mice shown in each plot are



grouped without indicating the age of the mouse for each curve. A general trend in the "pictures" of the enzyme actions with increase in age of the mouse is apparent.

oldest. The differences and changes are not as clear and definite as with the rats, but are still readily seen. The first group of curves, mouse ages 6 days before birth to 1 day before birth, approach the type found with embryo rats and a number of embryo rabbit tissues The low benzyl acetate values, one-half to one-third those of methyl butyrate, are significant. A certain amount of irregularity is, however, apparent with these curves In the next chart, the values of the two butyrates increase, while in the third, ages 7 to 9 days, a type which may be said to correspond to the adult mouse is apparent. This type or "picture" of the enzyme actions shows no real or constant change as the mice become older, although several changes with the oldest mice will be indicated, which, if borne out by other findings, may be rather significant

A study of the curves shows certain irregularities in the sense that the "pictures" at times do not fall into the sequence to be expected at the indicated ages of the mice in days There are more of such apparent exceptions than with the rats, where only isolated cases failed to conform to the general scheme It must be remembered, however, that, in dealing with biological individuals, it is impossible to control the conditions so as to obtain chemical uniformity if such is desired, and that as with the rats the results for every mouse whose age was definitely known has been included, none being omitted because of apparent failure to conform to a preconceived scheme As stated earlier, the mice used were not all of one strain There is an irregularity in the sequence to which attention will be called. In the seventh chart, mouse ages 48 to 50 days, there is a real reversion toward the embryo type. The curve showing this is the average of two independent but closely agreeing series for 48 day old mice. Because of the lack of data of the longevity of the strains of mice used in these experiments, it is difficult to state with any degree of certainty whether the ages attained with the oldest mice really correspond to so called "old age".

The results answer definitely the question raised in the introduction as to whether the youngest mice correspond in type to rat embryos or to mouse carcinomas. This is brought out by the curves shown in Fig. 2, where the mouse and rat embryo "pictures" are seen to be quite similar, the rat tumor "picture" not much different, but the

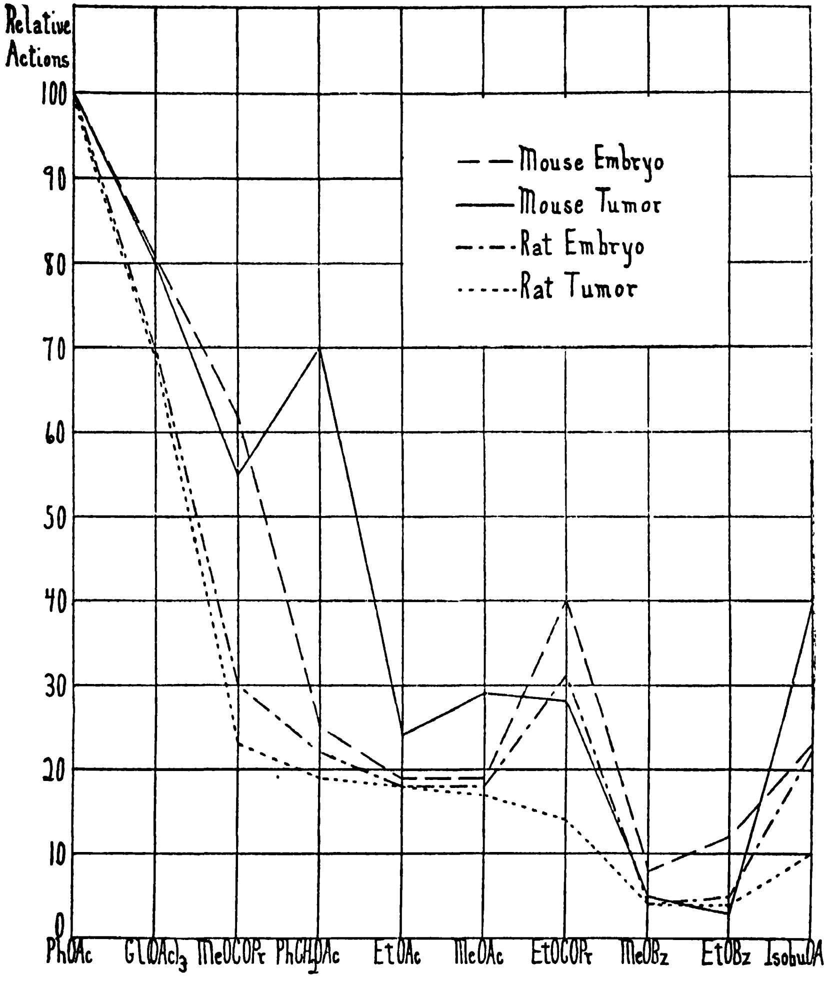


FIG. 2. The four curves of relative actions refer to the mouse embryo (6 days before birth), mouse tumor (averages of the Twort and Bashford 63 mouse carcinomas), rat embryo (averages of rats taken 3 days to 1 day before birth), and rat tumor (Flexner-Jobling rat carcinoma). The high value of benzyl acetate for the mouse tumor differentiates this curve sharply from the rest. The mouse embryo and mouse tumor have higher values for methyl butyrate than do the rat embryo and rat tumor; while the mouse and rat embryos have somewhat higher ethyl butyrate values than the mouse and rat tumors. On the whole, the curves for the mouse and rat embryos are quite similar, that for the rat tumor not greatly different, but that for the mouse tumor quite different.

mouse tumor "picture" very much different. The following relations also bring this out clearly. With the methyl butyrate-benzyl acetate actions, for the mice, the ratio of these actions did not change with increase in age, but oscillated about the general mean of 2.3 to 1. In other words, the value for methyl butyrate was from 1.5 to 3 times as large as that for benzyl acetate at every age. In comparison with this, for the Twort mouse carcinoma the average methyl butyrate value was 0.93 that of the benzyl acetate, while for the Bashford 63 mouse carcinoma, the corresponding value was 0.91. Also for the rat embryos, the methyl butyrate values were always considerably greater than the benzyl acetate values. These facts prove conclusively that the mouse embryos conform to the embryonic type found in other connections and differ from the two mouse tumor types which have been studied.

A number of regularities are readily observed if the results shown in Fig. 1 are studied carefully. A few of these will be pointed out. The ethyl butyrate values are lower than the methyl butyrate values throughout the life cycle of the mouse, their average value being very close to two-thirds of the latter. This may be compared to an average value very close to 1.00 for the rats of different ages, the methyl and ethyl butyrate values being practically the same. The average ethyl acetate-methyl acetate ratio was 1.14 for mice and 1.20 for rats for the different ages, while the average ethyl benzoate-methyl benzoate ratio was 0.88 for mice and very close to 1.30 for rats.

Because of the lack of marked change of the "pictures" of the enzyme actions of mice at different ages, the results will not be discussed further in detail.

Pregnancy had no influence on the enzyme "picture" of the mother mouse. Several were tested in this way and showed the "pictures" corresponding to the age of the otherwise normal mouse. This conclusion agrees with the results obtained with pregnant rats.

The absolute values of the ester-hydrolyzing actions are shown in Table I. The results for mice of the same or nearly the same ages are averaged and presented as one series of results. The actions increased on all the esters with increase in age of the mice, beginning with comparatively small values for the embryo, reaching high values

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Extracts of Whole Mice of Different Ages on the Indicated Esters.

Age	PhOAc	Gl (OAc)$_3$	MeOCOPr	PhCH$_2$OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
−6	1.67	1.34	1.03	0.42	0.32	0.32	0.68	0.14	0.20	0.38
−1, 0, 0 (Av.)	2.69	1.77	1.85	0.77	0.59	0.56	1.15	0.49	0.46	0.69
4	3.17	1.91	1.91	1.04	0.69	0.73	1.39	0.62	0.56	0.89
5	3.25	2.33	2.53	1.15	0.83	0.80	1.90	0.61	0.62	0.83
7	4.29	2.96	5.17	1.79	1.42	1.23	2.86	0.79	0.77	1.34
8, 9, 9 (Av.)	3.70	2.42	3.69	1.50	1.00	0.94	2.25	0.61	0.55	1.07
12	4.75	2.82	4.78	1.81	1.35	1.15	2.83	0.73	0.67	
15	4.20	2.86	4.21	1.68	1.30	1.26	2.54	0.65	0.56	1.28
17	4.48	3.12	4.14	2.80	1.36	1.10	2.90	0.76		
20, 20 (Av.)	6.31	3.30	4.78	2.20	1.62	1.50	3.37	1.06	0.94	1.56
23	5.51	3.26	4.78	1.90	1.54	1.32	3.10	0.88	0.72	1.14
27	4.79	2.62	3.48	1.63	1.18	1.00	2.46	0.78	0.64	1.34
29	5.38	3.12	3.69	2.26	1.42	1.25	2.88	0.96	0.86	1.64
39, 39, 40, 40 (Av.)	5.28	3.09	4.79	2.04	1.69	1.36	3.38	0.89	0.81	1.72
42	5.69	3.78	6.45	2.24	2.21	1.75	3.79	1.10	1.00	2.09
46 (Av. of 4)	5.31	3.10	4.66	1.97	1.52	1.31	3.02	0.87	0.77	1.69
47	5.23	2.94	4.60	2.03	1.61	1.37	3.04	0.84	0.75	1.63
48 (Av. of 3)	5.21	2.85	3.49	1.95	1.19	1.08	2.46	0.76	0.64	1.39
49, 50 (Av.)	5.89	3.40	5.19	2.34	1.57	1.44	3.21	0.99	0.78	1.99
52	6.15	3.57	5.65	2.26	2.09	1.84	4.00	1.16	0.90	2.18
53 (Av. of 2)	5.75	2.85	4.91	1.89	1.43	1.09	2.94	0.92	0.78	1.45
54, 55	4.98	3.02	3.95	2.06	1.48	1.28	2.88	0.89	0.80	1.79
59	5.21	2.92	4.58	1.93	1.39	1.17	2.90	1.01	0.74	1.58
71	6.38	3.67	5.73	2.45	1.84	1.65	3.40	1.14	0.91	2.16
89	5.77	3.33	4.55	2.02	1.33	1.26	2.84	1.02	0.74	1.76
113	4.76	2.58	4.14	1.91	1.35	1.15	2.78	0.74	0.65	1.53
161 (Av. of 2)	5.91	3.26	5.93	2.59	1.78	1.48	3.44	0.87	0.85	2.02
185, 191 (Av.)	5.74	3.29	5.02	2.12	1.77	1.45	3.42	0.90	0.79	2.10
241, 246 (Av.)	5.53	3.11	5.43	2.24	1.71	1.46	3.36	0.88	0.78	2.02
289	5.57	3.51	5.84	2.29	1.88	1.67	3.48	0.92	0.87	2.09
333	6.33	2.87	4.14	2.28	1.37	1.19	2.98	0.87	0.65	1.82
437, 452, 466 (Av.)	4.70	2.78	4.56	1.82	1.42	1.25	2.83	0.85	0.71	1.56
557	5.31	2.70	3.30	1.86	1.30	1.18	2.83	0.86	0.70	1.54
626	5.86	3.08	4.14	2.05	1.42	1.23	2.64	0.86	0.66	1.74

at the age of about 7 to 20 days, and then fluctuating irregularly about these.

These results may be compared with the absolute ester-hydrolyzing actions observed with whole rats of different ages. With the rat, the actions were small for the embryo, increased rapidly with increase in age to apparent maxima, and then decreased gradually as the rats became older. The small initial values followed by increases are similar for the mouse and the rat, but the markedly decreased actions on almost all the esters, as the animals aged, which were found with the rat were not observed with the mouse. This may have been due in part, perhaps, to the fact that the ages of the oldest mice were not as great relatively as those of the rats.

SUMMARY.

The ester-hydrolyzing or lipase actions of extracts of whole mice whose ages ranged from approximately 6 days before birth to 1 year 8 months 21 days were tested on ten simple esters by the method described in previous papers. The "pictures" of the relative enzyme actions changed from a type approaching the "embryonic" as found with embryo rats and a number of tissues of rabbit embryo, to a type characteristic of the adult mouse. The mouse embryos corresponded to the rat embryos in type and differed markedly from the mouse carcinomas which have been studied. The relative and absolute enzyme actions are discussed in some detail, and the results compared with the results obtained for the life cycle of the rat.

The writers wish to thank Mr. Edmond White for assistance in carrying out the experimental work.

AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

III. THE CONDUCTIVITY OF SULFANILIC ACID-LYSIN MIXTURES.*

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(Accepted for publication, July 26, 1926.)

The hypothesis that a mixture of two ampholytes behaves, within certain limits, as an individual (1) with characteristics distinct from either component can also be semiquantitatively tested by certain conductivity experiments. These are based on the observation that the addition of such a substance as gelatin markedly decreases the observed conductivity of a phosphate solution. The magnitude of the decrease is a function of the pH and passes through a minimum at the isoelectric point of the gelatin. In other words, the specific contribution of the gelatin to the measured conductivity is negative. Consider the case of two such ampholytes, A and B. Their separate effects on the conductivities of buffer solutions will be as above, and will be a minimum at their respective isoelectric points. When mixtures of the two are observed, however, their effect is altered due to the fact that through a certain pH range, namely between their respective isoelectric points, there will also be a tendency for mutual "binding" of A and B with a resulting "release" of buffer, resulting in a measured conductivity greater than would be calculated from a knowledge of their behavior when observed separately. This difference between the observed and calculated value of the conductivity may be expected to pass through a maximum at or near what has been termed the isoelectric point of the system.

In case the substances A and B have themselves conductivities comparable with those of the buffer, there is another possibility, which is much more probable, but which cannot be certainly predicted.

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* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 117.

Their specific contributions to the conductivity, when observed separately, may be positive or negative, or may even change sign at some definite pH. One might expect, however, that, if there be any considerable mutual binding tendency between two fairly conducting substances, the measured conductivity will be less at the isoelectric point of the system than would be expected from their separate behaviors, and that this difference would be a maximum at this pH.

To test this idea the conductivities of sulfanilic acid and of lysin were measured in phosphate buffers. The buffers were in all cases 0.02 molal in phosphate. The concentration of the sulfanilic acid and of the lysin were, throughout, the same, the former being 0.020 molal and the latter about 0.023 molal. The mixtures were 0.020 molal in sulfanilic acid and 0.023 molal in lysin. The water employed in the work had a specific conductivity of about 2×10^{-6} reciprocal ohms at room temperature.

The measured conductivities at 25°C., in reciprocal ohms, are given in Table I. The pH values were measured by means of the apparatus used in the titration work described in the preceding paper (1).

From these values pH-conductivity curves were plotted and the conductivities at comparable pH values were read off. These latter values are given in Table II. Obviously the same buffer mixture will not bring sulfanilic acid to the same pH that it will lysin when the buffer concentration is at all comparable to that of the sulfanilic acid or the lysin. It will be seen, however, that for any pH, with constant total-phosphate concentration, the concentrations, and thus the conductivities, of the various anion species of the buffer will be the same in all cases unless certain of them tend to be bound by the sulfanilic acid or the lysin. There will, however, be a difference in the sodium ion concentration, and some correction must be made for this. In Table II there is therefore included the total sodium concentration. The correction is made by referring to the concentration in the pure buffer at the same pH. For example, at a pH of 4 there was a concentration of 0.0197 in the buffer but of 0.0464 in the sulfanilic acid. To get the contribution of the sulfanilic acid itself, the measured conductivity of the buffer is subtracted from that of the sulfanilic acid and buffer at the same pH. This resulting conductivity is partly due to the sulfanilic acid, and partly due to the excess of sodium ion.

TABLE I.

Buffer		Sulfanilic acid plus buffer		Lysin plus buffer		Mixture plus buffer	
pH	*k*	*pH*	*k*	*pH*	*k*	*pH*	*k*
3.77	590	3.75	1105	3.75	543	3.90	1078
4.04	591	4.50	1134	4.24	564	4.34	1104
4.37	590	5.72	1200	4.87	592	4.74	1130
6.28	718	5.85	1208	5.26	630	5.30	1165
6.78	833	6.33	1251	5.65	709	5.75	1221
7.27	936	6.63	1306	5.88	762	5.91	1220
7.77	995	6.88	1355	6.36	831	6.00	1214
8.70	1010	7.12	1400	6.83	932	6.21	1230
9.75	1026	7.57	1457	7.40	1002	6.41	1287
10.27	1049	7.93	1487	7.95	1044	6.61	1345
10.70	1090	8.68	1510	8.43	1065	6.83	1383
11.07	1175	10.20	1550	8.83	1080	7.03	1405
		10.40	1565	9.23	1110	7.27	1434
		11.07	1677	9.72	1162	7.83	1496
				10.42	1270	8.36	1539
				11.00	1425	8.62	1555
						9.42	1622
						10.10	1717
						11.12	1965

TABLE II.

pH	Buffer		Sulfanilic acid			Lysin			Mixture		
	Na	*k*	*Na*	*k*	*k'*	*Na*	*k*	*k'*	*Na*	*k*	*k'*
4.0	.0197	590	.0464	1117	527	.0137	554	−36	.0403	1085	495
5.0	.0207	613	.0484	1166	553	.0153	601	−12	.0428	1147	534
5.2	.0209	623	.0485	1175	552	.0158	624	1	.0431	1164	541
5.4	.0215	635	.0485	1180	545	.0168	645	10	.0438	1170	535
5.6	.0220	649	.0486	1190	541	.0176	694	45	.04415	1196	547
5.8	.0231	665	.0496	1205	540	.0192	756	91	.0452	1226	561
6.0	.0243	684	.0506	1224	540	.02115	771	87	.0465	1214	530
6.2	.0258	706	.05144	1236	530	.0232	800	94	.0479	1228	522
6.4	.0276	739	.0525	1262	523	.0259	838	99	.0498	1286	547
6.6	.0297	785	.0537	1300	515	.0290	890	105	.0518	1344	559
6.8	.0320	835	.05525	1338	503	.0312	929	94	.0537	1382	547
7.0	.0343	880	.0570	1375	495	.0336	955	75	.0557	1402	522
8.0	.0393	1002	.0612	1489	487	.0389	1046	44	.0605	1511	509
9.0	.040	1013	.0625	1514	501	.0429	1090	77	.0645	1578	565
10.0	.0405	1035	.0632	1538	503	.0472	1199	164	.0695	1701	666
11.0	.0436	1165	.0660	1666	501	.0556	1425	260	.078	1933	768

The values of the conductivities must be multiplied by 10^{-6} to give reciprocal ohms.

The difference in total sodium concentration can be at once obtained, but some assumption must be made as to the relation of the total sodium concentration to that of the sodium ion. Due to the necessity of this correction, the values of these conductivities are not presented as significant data in themselves, in fact their precise magnitude as well as their real significance is a question. However, the general shape of the curve obtained by plotting them against the pH is considered significant, and slight errors in the sodium ion correction will alter neither the general shape of the curve nor the position of the maximum in Fig. 1. Assuming, then, the isohydric principle, and the value 0.92 for the degree of ionization of the sodium salts, the conductivity correction for the sodium ion can be calculated from its ion conductance at 25°C., 50.7, and the cell constant. Table II, then, gives the total sodium concentration, the measured total conductivity in the column headed *k*, and the apparent conductivity of the substances studied, uncorrected for sodium ion concentration difference, in the column headed *k'*. The latter value is merely the difference between the conductivity of the solution and that of pure buffer at the same pH.

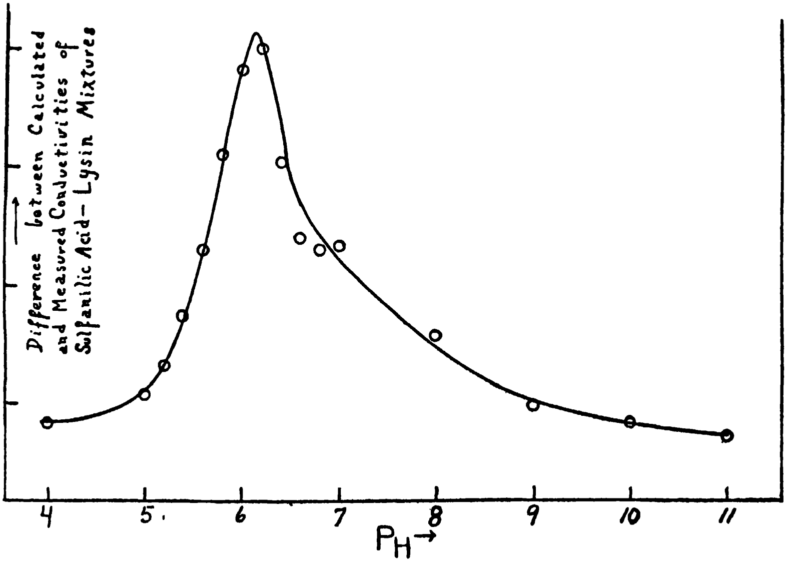

FIG. 1.

Table III gives the final corrected values for the contributions of sulfanilic acid, lysin, and their mixture to the total conductivity. The last column contains the differences obtained by subtracting the measured contribution of the mixture from the sum of the contributions of the two components when studied separately. These differences are plotted against pH in Fig. 1.

This curve, representing the pH function of the decrease in conductivity from what might be expected, due presumably to mutual binding of sulfanilic acid and lysin, passes through a fairly well defined maximum somewhere between the pH values 6.1 and 6.2. Calculation, by the method described in the preceding paper (1), of the value of the isoelectric point of this system, gives, using 7×10^{-4} for the acid ionization constant of sulfanic acid and 7×10^{-8} for the basic ionization constant of lysin, a pH of 6.03. The agreement seems quite satisfactory, considering the method of obtaining the experimental data and the problematical value of the basic ionization constant of lysin.

TABLE III.

pH	$k \times 10^6$				
	Sulfanilic acid	Lysin	Mixture (observed)	Mixture (calculated)	Difference
4.0	157	48	209	205	−4
5.0	167	63	227	230	3
5.2	169	72	231	241	10
5.4	170	75	225	245	20
5.6	172	106	239	278	39
5.8	172	145	254	317	63
6.0	175	131	222	306	84
6.2	174	130	215	304	89
6.4	177	123	239	300	61
6.6	179	115	252	294	42
6.8	180	105	246	285	39
7.0	180	85	225	265	40
8.0	183	50	215	233	18
9.0	188	37	225	225	0
10.0	188	71	263	259	−4
11.0	190	93	290	283	−7

DISCUSSION.

While the above experiments were made on a comparatively simple system in order that a somewhat more definite interpretation might be possible, the more interesting and perhaps more obvious applications of the results are in connection with the much more complicated, though in many respects similar, systems which go to make up biological tissues

One of the striking apparent anomalies which the point of view developed in this series of papers tends to straighten out is brought out in Fig 2 From water absorption and behavior toward dyes Robbins (2) has found for the complex system potato tuber an isoelectric point at a pH of about 6, depending somewhat on the buffer used for adjusting the pH (Curve *C*). That this value is not even approximately characteristic of the protein most commonly associated with potato, namely tuberin, is apparent from the work of Cohn, Gross, and Johnson (3), who found for this protein an isoelectric point at a pH of about 4 Their tuberin was obtained from acid precipitation of potato juice It is significant to note that they describe the precipitation of protein from potato juice by alkali as well as by acid. The latter precipitation reached a maximum at a pH of about 8, but was not otherwise studied. The solubility curve for the protein material in potato juice as a function of pH is given by Curve *A* of Fig. 2. (Curve *B* gives the same for carrot juice indicating similar behavior.) Both are taken from the work of Cohn, Gross, and Johnson. The point of maximum solubility between the two minima corresponds roughly with Robbins' isoelectric point of the system potato tuber, *i.e.* with the point of minimum water imbibition (Curve *C*). The two points are not exactly the same, but Robbins was working with whole tissue, while Cohn, Gross, and Johnson were working with the extracted juice.

The comparatively large specific effect of the particular buffering material employed on the isoelectric point of a complex system may also be expected, due to selective "binding tendencies" between the specific buffer ions and one or another of the components of the original system. Thus Robbins (2) finds a difference of nearly half a pH unit between the isoelectric points of potato tuber tissue as determined by using citrate or phosphate buffer and as determined by phthalate buffer. In Fig. 2, Curve *C*, Curve I was obtained using phosphate adjustments and Curve II using phthalate adjustments.

The concept of such a mixed system offers also a possible chemical mechanism for the taking on of foods of both a basic and an acidic nature. Thus, in the case of a complex system, there will be a fair pH range through which one or the other of the components will exhibit a tendency to bind foods either of a carbohydrate or of a peptone nature. Growth curves over a fair pH range are suggestive on this point

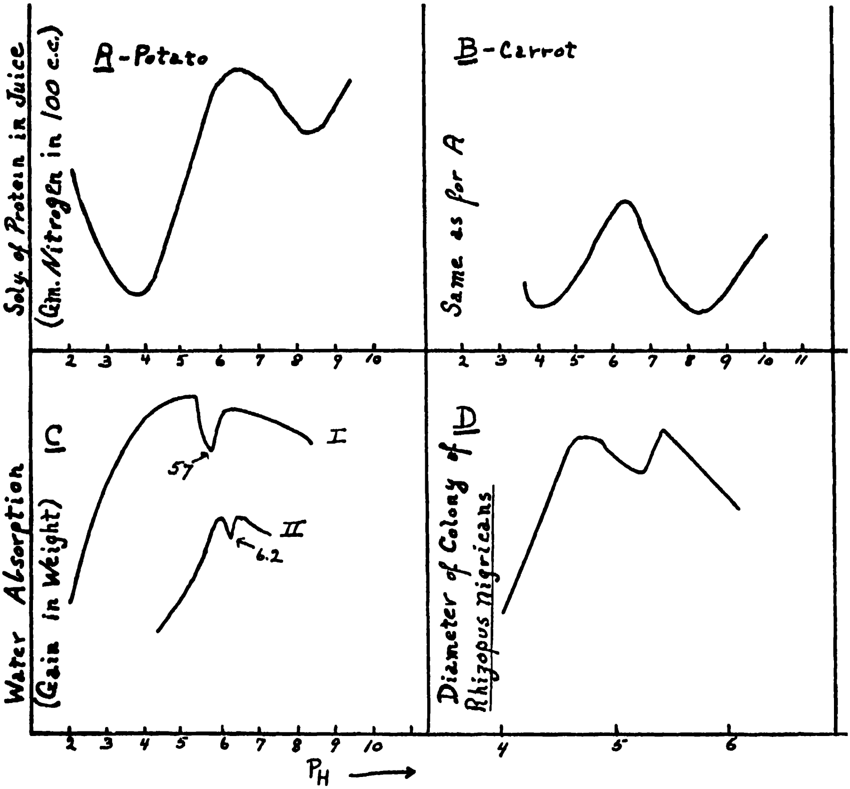

FIG. 2. Curve *A*, solubility of protein material in potato juice; Curve *B*, solubility of protein material in carrot juice (3); Curve *C*, water absorption by potato tuber tissue (2); Curve *D*, growth curve of *Rhizopus nigricans* on potato dextrose agar (4).

(Fig. 2, Curve *D*) (4). At quite low or quite high pH values, nutrition, according to these curves, is very inefficient Starting in acid solution, as the pH increases the rate of growth at first steadily increases. If the taking on of foods is primarily influenced by the ionic condition of the organism rather than by the ionic condition of the foods, and if the organism were acting as a simple ampholyte, we might expect an optimum condition for growth at its isoelectric point. (This would not be analogous to water imbibition) At such a pH, in case of a simple ampholyte, the active anion concentration would be equal to the active cation concentration. In a mixed system, however, such is not the case when the lower isoelectric point, *i.e.* of one of the components, is reached, and actually the growth curve continues to rise, probably until the extent of mutual binding of the components of the system itself begins to affect results The curve thus passes through a maximum and then descends to a minimum, probably at or near the point of maximum binding, *i e* the isoelectric point of the system The rate of growth, even at this minimum, is higher than it is at those points corresponding more probably to the isoelectric points of the components, and the fact that it is a distinct minimum does not at all mean that growth is poor From this minimum point, as one proceeds to higher pH values, the curve again rises, passing through another maximum, and then rapidly falls.

Work is now in progress to determine, if possible, the pH growth curves of organisms utilizing foods which might be considered entirely acidic in character, as well as foods which are entirely basic. Experiments on the specific effect of individual buffers are also under way, and it is hoped that soon the point of view developed here can be somewhat quantitatively tested out on systems somewhat more complicated than those herein described, but which are still sufficiently definitely known to permit of quantitative study and interpretation

SUMMARY.

Conductivities of sulfanilic acid, lysin, and mixtures of the two were made over a wide pH range, the pH being adjusted by means of phosphate buffers The actual conductivities of the sulfanilic acid, the lysin, and the mixture were calculated. The difference between the conductivity of the mixture and the sum of the conductivities of the components alone passes through a maximum at a pH theoretically calculable as the isoelectric point of the system.

Certain applications of the results are made to the explanation of the behavior of living tissues.

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AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

IV. Note on the Isoelectric Point and Ionization Constants of Sulfanilic Acid.*

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(Accepted for publication, July 26, 1926.)

In the experiments described in the preceding papers the assumption was made that sulfanilic acid was an ampholyte. While its formula would tend to justify such an assumption, there seems to be no record of any determinations of its basic ionization constant or of its isoelectric point. It is to be expected that the magnitude of the former is very small and that the isoelectric point will be at a very low pH.

Titration methods do not offer a satisfactory method of attack since the pH at which basic neutralization commences is so low that a small change in E.M.F. corresponds to a relatively large difference in hydrogen ion concentration, and moreover the hydrogen ion concentration change is not a measure of salt formation in such a region of pH. Also the large acid ionization constant of the sulfanilic acid tends to "cover" or mask any small basic neutralization.

Solubilities were therefore determined in water and in various concentrations of hydrochloric acid. Table I gives results at 24°C., which are typical. The first eight columns give the molalities of the substances named. The basic ion from the sulfanilic acid is called S^+, and the acid ion S^-. K, in Column 9, is the function $\dfrac{(\mathrm{H}^+)\begin{pmatrix}\text{unionized}\\ \text{sulfanilic}\end{pmatrix}}{(\text{salt})}$. The degree of hydrolysis, h, is calculated for a salt concentration equal to the concentration of HCl corresponding to its place in the table.

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* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 118.

Values of *h* are calculated by taking a total salt concentration equal to the corresponding hydrochloric acid concentration and, using the value of *K* in Column 9, solving for the concentration of unhydrolyzed chloride. We are in such a pH region, however, that though we can neglect the acid ionization of sulfanilic acid, we cannot neglect the ionization of the HCl formed by hydrolysis; so that, since the hydrolysis is in all cases large, the measured value of the hydrogen ion con-

TABLE I.

1 HCl	2 $H^+ \times 10^3$	3 $OH^- \times 10^{12}$	4 Solubility of sulfanilic acid	5* $S^- \times 10^4$	6† $S^+ \times 10^4$	7 Unionized sulfanilic acid	8‡ Salt $\times 10^3$	9§ *K*	10‖ *h*	11‖ $K_b \times 10^{15}$
.00	.67	15.	.0715	56.	.93	.0648	—	—	—	—
.05	4.7	2.1	.0664	9.8	6.3	.0648	—	—	—	—
.07	6.5	1.54	.0661	7.0	8.5	.0646	—	—	—	—
.08	7.4	1.35	.0663	6.2	9.6	.0647	—	—	—	—
.087	8 0	1.25	.0665	5.8	10.5	.0649	—	—	—	—
.10	9.2	1.08	.0675	5.1	—	—	1.72	3.46	.97	2.6
.25	22.5	.44	.0689	2.1	—	—	3.77	3.92	.948	2.3
.50	42.	.24	.0709	1.2	—	—	5.9	4.6	.923	1.8
									(.914)	(2.1)
.85	68.	.147	.0714	.73	—	—	6.5	6.75	.917	1.2
									(.874)	(1.95)
Mean						.06476				
1.			.0713							
1.3			.0705							
1.8			.0668							
2.			.0659							
2.5			.0645							
4.			.058							
6.			.051							

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* Using the value 7×10^{-4} for the acid ionization constant.

† Using the value 2×10^{-15} for the basic ionization constant.

‡ Obtained by subtracting the mean of the values in Column 7 plus twice the respective values in Column 5 from the values in Column 4.

§ The indicated function of the values in Columns 2 and 8 with the mean of those in Column 7.

‖ The values in parentheses are obtained by putting *K* (Column 9) equal to 4.0. See text.

centration for the corresponding molality of HCl is substituted in the formula rather than the total concentration of the HCl formed by hydrolysis.

It will be noted that the values of K in Column 9 regularly increase. This is due to the fact that at higher concentrations of HCl the actual solubility of the sulfanilic acid decreases rapidly as the HCl concentration increases, as shown in Table I. At these higher HCl concentrations we are in such a pH region that the thermodynamic environment of the solvent changes rapidly with comparatively small changes in the ratio of the hydrogen ion concentration. For this reason it is thought that a value of about 4.0 for K in Column 9 is probably not far from correct, and the corresponding values of h and K_b are accordingly given in parentheses.

An approximate check on the magnitude of K_b can be obtained by noting the fact that there is a point of minimum solubility which comes apparently between cH values 0.047 and 0.065. Using the well known Michaelis relation, and the value 7×10^{-4} for K_a, K_b is found to lie between 1.7 and 3.2×10^{-15}.

Other Constants Used in the Series.

From the titration curves in Paper II of this series the pH at which any fraction of glycine or sulfanilic acid is neutralized may be read. This permits a calculation of their ionization constants, values of which are given in Table II.

These values are in good agreement with those given by Scudder (1) for glycine. The curve for sulfanilic acid did not permit as satisfactory reading, and since the literature gives values which seem to vary somewhat, the following determination of the acid ionization constant of sulfanilic acid was made. The method was to take conductivity ratios at different known concentrations. Since conductivities are additive, their ratios will be a measure of the ratios of the sulfonate ion concentrations at respective total acid concentrations. Thus if X be the concentration of sulfanilic ion (and also of hydrogen ion) at total concentration C, and X' be their concentrations at total concentration C', then $X' = rX$, where r is the ratio of the conductivities at concentrations C and C'.

TABLE II.

Ion/molecule ratio	Glycine				Sulfanilic acid	
	cOH × 10^{12}	K_b × 10^{12}	cH × 10^{10}	K_a × 10^{10}	cH × 10^4	K_a × 10^4
1 : 3	6.45	2.15	6.6	2.2	—	—
1 : 2	4.26	2.13	4.46	2.23	14.1	7.05
1 : 1	2.24	2.24	2.24	2.24	7.24	7.24
2 : 1	1.1	2.2	1.15	2.3	—	—
3 : 1	.76	2.28	.79	2.37	—	—
Mean...............		2.2		2.27		7.15

TABLE III.

Bottle	Total concentration sulfanilic acid	Resistance	Conductivity × 10^6
1	.05	1736	576.0
2	.01	4175	239.5
3	.005	6234	160.4
4	.0025	9513	105.1
5	.001	17391	57.5
Combination	**Ratio**	X × 10^3	K_a × 10^1
1–2	2.405	2.321 sulfanilic ion concentration Bottle 2	7.02
1–3	3.591	1.5565 " " " " 3	7.04
1–4	5.4805	1.021 " " " " 4	7.05
1–5	10.02	.5576 " " " " 5	7.03
2–3	1.493	1.5565 " " " " 3	7.04
2–4	2.279	1.0235 " " " " 4	7.10
2–5	4.165	.5573 " " " " 5	7.02
3–4	1.526	1.024 " " " " 4	7.11
3–5	2.790	.5606 " " " " 5	7.16
4–5	1.828	.5561 " " " " 5	6.97
Mean..			7.05

The value 7 × 10^{-4} has been used throughout.

One can thus obtain the two equations;

$$\frac{X^2}{C - X} = K, \quad \text{and} \quad \frac{(rX)^2}{C' - rX} = K$$

which can be solved simultaneously for X and K.

Table III gives the values obtained. The water used had a specific conductivity of .5 × 10^{-6} reciprocal ohms, and the temperature was 21.00°C. ± 0.02°, since it was at that temperature that the titration work had been largely carried out.

SUMMARY.

From the solubility minimum the value of the basic ionization constant of sulfanilic acid is shown to lie probably between the values 1.7×10^{-15} and 3.2×10^{-15}. From solubility measurements the value of this same constant is shown to lie probably between 2.0 and 2.2×10^{-15}, and the isoelectric point of sulfanilic acid is thus at a cH of 0.056 or a pH of 1.25. From conductivity ratios the acid ionization constant of sulfanilic acid is shown to be 7.05×10^{-4} at room temperature (21°C.).

Calculations are made, from data published in preceding papers, of the ionization constants of glycine, K_a being 2.3×10^{-10}, and K_b being 2.2×10^{-12}.

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THE OXIDATION-REDUCTION POTENTIAL OF THE LUCIFERIN-OXYLUCIFERIN SYSTEM.

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(Accepted for publication, October 27, 1926.)

Oxygen is necessary for the luminescence of the ostracod crustacean, *Cypridina*, and most luminous animals. A substance, luciferin, is oxidized to oxyluciferin in the presence of a catalyst, luciferase. The oxyluciferin can be reduced to luciferin again, so that one is justified in speaking of a luciferin $\leftrightarrows$ oxyluciferin system. When the words luciferin, oxyluciferin, and luciferase are used in this paper, I refer only to the crude impure solutions from *Cypridina hilgendorfii*. For the preparation and isolation of these substances the reader is referred to my book (1920) or earlier papers (1919, *b*).

We should like to know more about the nature of the oxidation of luciferin. First, it must be understood that the word oxidation is used for a number of quite different processes. We may recognize at least two distinct types of *reversible* oxidations.

(1). The oxidation of hemoglobin to oxyhemoglobin, in which the oxygen is actually bound, but very loosely. This well known change depends on the concentration or pressure of the oxygen and is more properly called an oxygenation than an oxidation. Such oxygenations do not possess an oxidation-reduction potential. Luciferin is not to be compared with hemoglobin because removal of the oxygen from an oxyluciferin solution will not bring about the formation of luciferin, as it will the formation of hemoglobin from oxyhemoglobin.

(2). The oxidation of hemoglobin to methemoglobin, in which no oxygen is actually bound in the methemoglobin molecule although oxygen may be used up in its formation. However, the reaction may go on in complete absence of oxygen, by oxidation with $K_3Fe(CN)_6$. Conant (1923–25) and his coworkers have thrown new light on this system, which indicates that it has a definite oxidation-reduction potential. If hemoglobin in acid solution is represented by $H_3Hb^{\equiv}$, then oxyhemoglobin is $H_3HbO_2^{\equiv}$ and methemoglobin is $H_2Hb^{=}$. A change in valence is the essential difference in the hemoglobin-methemoglobin transformation.

Other oxidations in which the essential change is one of valence necessitate also the removal of hydrogen, such as the oxidation of a leuco dye to the dye itself. Oxygen plays the rôle of hydrogen acceptor, forming water. Thus, methylene white (leucomethylene blue), MH_2, and safranin white (leucosafranin) oxidize to methylene blue, M, or safranin spontaneously in the air by loss of hydrogen;—$MH_2 + O = M + H_2O$. As Clark and his coworkers (1923–26) have shown, these reactions have a definite oxidation-reduction potential also.

I have previously pointed out (1918–19, *a*) that the oxidation of luciferin bears some resemblance to the oxidation of a leuco dye and presumably has an oxidation-reduction potential. The equation might be represented thus:

$$LH_2 \text{ (luciferin)} \leftrightarrows L \text{ (oxyluciferin)} + H_2$$
$$H_2 + O = H_2O.$$

For a more complete discussion of oxidation-reduction potential the reader is referred to the papers of Clark and collaborators (1923–26), Conant (1926), and Conant and Cutter (1924). Suffice it to say here that oxidizing and reducing agents can be arranged in a series of varying strength, with strongest reducing agents at one end and strongest oxidizing agents at the other The strength is conveniently measured by the potential which the oxidizing or reducing agent gives in contact with some noble indifferent metal, like gold or platinum. Reproducible potentials are observed only if the reaction (reductant $\leftrightarrows$ oxidant) is reversible, and the exact value varies with the H ion concentration and the ratio of oxidized to reduced substance. The symbol, E_0', is used for the potential of such an oxidizing agent as methylene blue at a definite pH and with chemically equivalent amounts ([red.]/[oxid.] = 1) of methylene blue (oxidant) and methylene white (reductant) in solution. The accompanying table gives the E_0' values for a number of oxidizing or reducing agents at a pH = 7.7, a favorable value for luminescence of luciferin.

The H electrode represents the reducing power of hydrogen gas under one atmosphere pressure in presence of a catalyst like finely divided platinum. Many reducing agents like sulfides and chromous chloride stand above the H electrode and hydrosulfites and titanous salts are near it. The oxygen electrode represents the oxidizing power of oxygen under conditions similar to those of the H electrode. Many oxidizing agents like permanganates and dichromates come below the oxygen electrode.

TABLE I.

E'_0 Value at pH = 7.7.

H_2 electrode	−.46
$Na_2S_2O_4$ (Na hydrosulfite or hyposulfite)	−.32 (approximately)
Safranin	−.30 "
Anthraquinone β Na sulfonate	−.26
Anthraquinone 2-6-di Na sulfonate	−.22
Indigo monosulfonate	−.20
" disulfonate (indigo carmine)	−.15
" trisulfonate	−.115
" tetrasulfonate	−.075
Methylene blue	−.01
1-4-napthoquinone	+.03
Lauth's violet	+.04
Methemoglobin (anaerobic)	+.08 (approximately)
1-2-napthoquinone	+.13 "
2-6-dichlorindophenol	+.20
Quinhydrone (equimolecular hydrochinone and quinone)	+.24
K ferricyanide	+.43
O_2 electrode	+.76

Where does the luciferin-oxyluciferin system stand in this scale? One may gain some idea of the position of the luminescent system in the potential scale by finding what substances, whose potentials are known, will oxidize luciferin or reduce oxyluciferin in absence of oxygen. We know that oxygen and platinum will oxidize luciferin and that hydrogen and platinum will reduce oxyluciferin (Harvey, 1923), so that the position of luciferin-oxyluciferin must lie between the oxygen and hydrogen electrodes. Some years ago (January, 1922) I attempted to oxidize luciferin *with light production in absence of oxygen* by cystine, methylene blue, quinone, potassium ferricyanide, and other substances, but always with negative results, as far as the appearance of luminescence is concerned.

Recently I have confirmed these results. I have also carried out a more systematic study to limit the potential of the luciferin-oxyluciferin system. It appears that luciferin may be readily oxidized in absence of oxygen *although no luminescence occurs*. It is all important to distinguish between the oxidation of luciferin with luminescence and without luminescence. Oxidation without luminescence may occur in absence of gaseous oxygen or in absence of luciferase (with gaseous oxygen)[1] but oxidation with luminescence never occurs without oxygen and without luciferase. I shall return to this point later.

Rapid oxidation of luciferin without luminescence can be demonstrated very clearly and simply by adding to a luminescent mixture of luciferin and luciferase in phosphate buffer (pH = 7.7), weak $K_3Fe(CN)_6$ solution drop by drop. On sufficient addition of $K_3Fe(CN)_6$ the luminescence will disappear *without any previous increase in brightness*. All the luciferin has been oxidized by the ferricyanide. On now adding weak sodium hydrosulfite ($Na_2S_2O_4$) solution to the mixture and then shaking with air, the luminescence will return. $Na_2S_2O_4$ has reduced the oxyluciferin formed by the ferricyanide, and with luciferase and oxygen present light appears. The oxidation and reduction can be brought about many times by successive additions of ferricyanide and hydrosulfite, so there can be no possibility that these reagents injure the luciferase.

It is obvious that one might titrate luciferin quantitatively with ferricyanide, using luminescence with luciferase as the end-point indicator, but some experiments along these lines show that there is no sharp end-point and lead me to believe that little significance is to be attached to such figures until luciferin can be obtained in the pure state. There are probably other easily oxidizable substances in my solutions besides luciferin.

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[1] The autoxidation of luciferin by oxygen in absence of luciferase might be compared with the autoxidation of benzaldehyde by oxygen. It is interesting to note in this connection that small amounts of hydrochinone or diphenylamine, which so markedly prevent (negative catalysis) the oxidation of benzaldehyde, have practically no inhibiting effect on the oxidation of luciferin, as judged by the luminescence. Quinone in the hydrochinone accelerates the spontaneous oxidation of luciferin. KCN, which inhibits respiratory oxidations, does not affect the luminescence of luciferin.

The experiment with ferricyanide and hydrosulfite shows at once that the potential of luciferin-oxyluciferin lies between these two substances. Since the oxidized form of any substance lower in the series of Table I will oxidize the reduced form of anything above, while the reduced form of anything above will reduce the oxidized form of anything below, we have only to test oxidation of luciferin and reduction of oxyluciferin by appropriate agents in the table.

There is only one point to guard against. If two substances lie close together in potential, the reaction between them will not be complete, for the potentials given are for equimolecular parts of oxidant and reductant, [red.]/[oxid.] = 1. For a ratio of [red.]/[oxid.] = 1/99 the potential would be shifted 50 millivolts toward the positive side, while for a ratio of [red.]/[oxid.] = 99, the potential would be shifted 50 millivolts toward the negative side. There is thus a certain band, about .1 volt wide, where oxidation and reduction will not be complete. By selecting substances in Table I fairly well separated from each other, we can limit the potential of luciferin. I have taken quinhydrone, 2-6-dichlorindophenol, 1-4-napthoquinone, methylene blue, indigo tetrasulfonate, indigo disulfonate, anthraquinone β Na sulfonate, anthraquinone 2-6-di Na sulfonate, and safranin and carried out the experiments in the following manner.[2]

Two test-tubes, *A* and *B*, are connected as shown in Fig. 1 by glass tubes through rubber stoppers. The tube, *C*, has an enlargement at *D* holding a small perforated platinum disk above which asbestos may be packed to serve as a filter. In testing for reduction of oxyluciferin, oxyluciferin + luciferase in M/10 phosphate (pH = 7.7) buffer is placed in *B* and the reducing substance, say dilute safranin in M/10 phosphate (pH = 7.7) buffer + some platinized asbestos in *A*. A stream of pure hydrogen freed of oxygen by passage over red hot platinized asbestos in a quartz tube, is then passed through the tubes, *A* and *B*, for ½ hour or more. The safranin is reduced to colorless safranin white by the hydrogen-platinum and all oxygen driven from the system. Then Tube *A* is inverted and its contents filter into *B*, the platinized asbestos being kept back by the asbestos filter. This is important since oxyluciferin is reduced by hydrogen-platinum.

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[2] I am deeply indebted to Dr. Keith Cannan for samples of quinhydrone, dichlorindophenol, and indigo tetrasulfonate and to Dr. J. B. Conant for samples of the napthoquinones and the anthraquinone sulfonates.

After mixing, oxidation of safranin will be indicated by reddening of the safranin white in the hydrogen atmosphere and reduction of oxyluciferin will be indicated by luminescence when air is admitted to the tube. There is undoubtedly some reduction of oxyluciferin by reduced safranin (which becomes red in the hydrogen atmosphere after mixing) and the anthraquinones, since luminescence is obtained when air is admitted to the mixture in *B*. A control tube of oxyluciferin and luciferase, but without addition of safranin white, or reduced anthraquinones in a hydrogen atmosphere gives no luminescence when air is admitted. Of the remaining dyes, indigo disulfonate, tetrasulfonate, methylene blue, 1-4-napthoquinone, and dichlorindophenol bring about practically no reduction of oxyluciferin. This is the case when the solutions have been in contact for 5 minutes before admitting air. If allowed to stand 90 minutes before testing, reduced indigo tetrasulfonate may reduce slightly, as judged by a very faint luminescence. However, the reduced yellowish indigo tetrasulfonate does not become appreciably colored (as would occur if it was oxidized by oxyluciferin) even after 90 minutes contact, so that the slight reduction of oxyluciferin can be detected only because a very small amount of luciferin gives a sufficiently bright luminescence to be detected by the eye. In other words the luminescent test is an extremely delicate

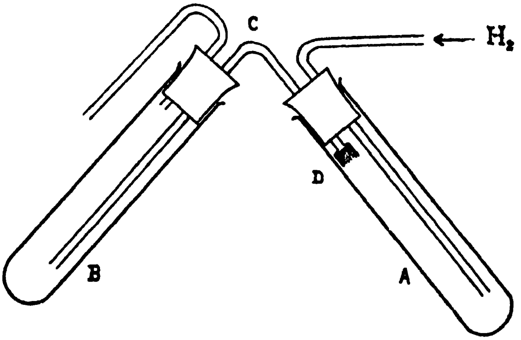

FIG. 1.

one. I am somewhat in doubt as to the meaning of very faint luminescences since a few bacteria may have brought about the slight reduction on standing 90 minutes. Relying on experiments which show undoubted reduction, I am inclined to place the luciferin-oxyluciferin system in the region between anthraquinone 2-6-di Na sulfonate (−.22) and indigo disulfonate (−.15).

Attacking the problem from the other side, that of oxidation, we place in Tube *B* luciferin in phosphate buffer (pH = 7.7) and in Tube *A* oxidized dye in phosphate buffer (pH = 7.7). Tube *C* need not be a filter in this case. After a stream of pure hydrogen has been passed for ½ to 1 hour, *A* is mixed with *B*. If oxidation of luciferin has occurred, the dye should become colorless and the luciferin give no more light on mixing with luciferase in air. The latter change is the best test since other reducing substances than luciferin may be present in crude luciferin solution. Passing up the list of substances in Table I from the oxidized end, there is no doubt that quinhydrone will oxidize luciferin in absence of oxygen but dichlorindophenol has practically no action and 1-4-napthoquinone or methylene blue none. Judging from these experiments on oxidation of luciferin, the potential of the luciferin-oxyluciferin system would be somewhere between dichlorindophenol ($E_o' = +.2$) and quinhydrone ($E_o' = +.24$).

The attempt to approach the potential from the oxidation and reduction side gives rather divergent values. There is thus no very sharp potential that can be assigned to luciferin. By this method of "bracketing" we find the limits for a pH = 7.7 somewhere between −.22 and + .24 volt. Systems between these values neither reduce oxyluciferin nor oxidize luciferin. Perhaps that is due to poising[3] action in a mixture of oxidizable and protein substances, for it must not be forgotten that luciferin solutions contain many other substances from the luminous animal besides luciferin.

If impurities are not obscuring the result, the luciferin-oxyluciferin system behaves like some of those described by Conant, which are irreversible. I say irreversible, although it is quite certain that the stronger reducing agents which lie near the hydrogen electrode will reduce readily. These include hydrosulfites, sulfides, chromium chloride ($CrCl_2$), titanous chloride ($TiCl_3$), and hydrogen formed at cathodes or from metals (aluminum amalgam and magnesium in ammonium salts are especially good methods) or in contact with Pt or Pd. At the same time it is just as certain that quinone, K ferricyanide, Br water, and strong oxidizing agents will oxidize luciferin. We are not dealing with such irreversible oxidations, as in the case of aliphatic aldehydes to acids, where the acid cannot be reduced to aldehyde no matter how strong the reducing agent; or such irreversible reductions, as in the case of dibenzolylethylene to dibenzolylethane, where the latter cannot be oxidized no matter how strong the oxidizing agent (Conant, 1926).

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[3] Similar to buffer action in acid-alkaline solutions (see Clark, 1923–26).

There is a certain range in which neither oxidation of luciferin nor reduction of oxyluciferin occurs. Perhaps we are justified in speaking of both apparent oxidation and apparent reduction potentials, the "apparent reduction potential" differing from the "apparent oxidation potential" (in Conant's sense, 1926) by about .5 volt.

The preceding experiments also prove that oxidation of luciferin may occur without oxygen (and without luminescence). What are the conditions for oxidation with luminescence? We know that luciferase is necessary for luminescence. Are *both* luciferase *and* oxygen necessary, or will luminescence appear in presence of luciferase alone, if oxidation of luciferin is brought about by $K_3Fe(CN)_6$? It is found that both luciferase and oxygen are necessary, for on adding potassium ferricyanide solution free of oxygen to a mixture of *luciferin and luciferase in absence of oxygen* (and of course dark) no luminescence will appear, although the luciferin will be oxidized, as is proved by the absence of light when oxygen is later admitted to the vessel.

We may suppose that the oxidation of luciferin by oxygen at the surface of colloidal particles of luciferase results in luminescence. The light is characteristic of the luciferase rather than the luciferin, since the color of the luminescence may be shown to be dependent on the kind of luciferase used and not on the luciferin (Harvey, 1917, 1924, *a*). Will any other easily oxidizable compounds luminesce when oxidized by oxygen *in presence of luciferase?* All my attempts to find such substances have failed, although I have tested many hydroxy- and aminophenols, leuco dyes, cysteine, and reduced bodies of unknown composition formed by reduction in tissue extracts of various animals, yeast and bacterial cultures.

For luminescence of *Cypridina* it is therefore necessary to have luciferin, luciferase, and free oxygen dissolved in water. It makes no difference how rapidly luciferin is oxidized by oxygen (for instance at high temperatures), luminescence never appears unless luciferase is also present, nor will luminescence appear in presence of luciferase unless oxygen is the oxidizing agent. Therefore, high reaction velocity *per se* is not a necessity for luminescence. But if luciferase and oxygen are present, then the greater the reaction velocity, the brighter will be the luminescence (Amberson, 1922).

SUMMARY.

The oxidation-reduction potential of the *Cypridina* luciferin-oxyluciferin system determined by a method of "bracketing" lies somewhere between that of anthraquinone 2-6-di Na sulfonate (E_o' at pH of 7.7 = $-.22$) which reduces luciferin, and quinhydrone (E_o' at pH of 7.7 = $+.24$), which oxidizes luciferin. Systems having an E_o' value between $-.22$ and $+.24$ volt neither reduce oxyluciferin nor oxidize luciferin. If the luciferin-oxyluciferin system were truly reversible considerable reduction and oxidation should occur between $-.22$ and $+.24$. The system appears to be an irreversible one, with both "apparent oxidation" and "apparent reduction potentials" in Conant's sense. Hydrosulfites, sulfides, $CrCl_2$, $TiCl_3$, and nascent hydrogen reduce oxyluciferin readily in absence of oxygen but without luminescence.

Luminescence only appears in water solution if luciferin is oxidized by dissolved oxygen in presence of luciferase. *Rapid* oxidation of luciferin by oxygen without luciferase or oxidation by $K_3Fe(CN)_6$ in presence of luciferase but without oxygen never gives luminescence.

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GALVANOTROPISM AND "REVERSAL OF INHIBITION" BY STRYCHNINE.

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I.

Definite neuromuscular effects are produced in a variety of organisms by the passage of a constant electric current. The character of the induced galvanotropic curvatures and movements has played a certain part in the development of the tropism doctrine (*cf.* Loeb, 1918), but these reactions still await quantitation—which is difficult—as well as fuller utilization for the analysis of animal movements. The interpretation of galvanotropism in metazoans turns at present upon the necessary and sufficient assumption that the current serves to excite definite groups of nerve cell bodies (presumably as determined by the axial orientation of these cells with respect to the polarity of the current—Loeb and Maxwell, 1896; Loeb, 1918; Moore, 1922–23, *b*).[1] The effects are such that among annelids, for example, the animal typically extends and lengthens when the head is toward the cathode, but shortens when the current is in reverse direction. These effects are in certain respects similar to those produced by neurophil drugs. Strychnine, for example, induces a similar and comparable elongation, whereas nicotine leads to shortening. It was considered that if the effects of such alkaloids are indeed due to selective or differential unions with particular groups of nervous elements, the result of combining the action of a substance of this type with that of the electric current should be merely an accentuation of the primary action, since the response induced involves activity of the same nerve-muscle groups. In certain cases this turns out to be true.

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[1] It should be noted, however, that the apparent impermeability of cells to direct current imposes certain conditions on the form which this interpretation may take.

The effect of strychnine is of especial interest. In the spinal cord of vertebrates it is usually supposed (Starling, 1926) that the strychnine effect is due to abolition of the inhibitory component of normal coordination, so that the inhibitory effect is transposed into an excitatory one. Coordination thus becomes impossible, for antagonistic muscles contract together, so that the resulting movement is determined by that muscle group which is the stronger. The locus of this action is commonly assumed to be at synapses (or their homologues), but until the nature of inhibition is better understood it might be assumed that the strychnine effect may really involve the elective excitation of nerve cell bodies.

Reversal of reaction by strychnine is apparent in a variety of invertebrates[2] in which the coordination of antagonistic muscle groups is requisite for normal movements. If the effect of the strychnine is primarily one depending upon the elective excitation of particular nerve cells, then a strychninized annelid, in which the longitudinal muscles are relaxed, circular muscles contracted, so that the animal is much elongated, should be caused to elongate still further when submitted to the action of a constant current with cathode at the head. The fact is that under these conditions the usual cathodic galvanotropism is reversed; the animal shortens when the head is toward the cathode, elongates when the head is toward the anode. The simplest interpretation of this result is that the reversal effect of strychnine is a true synaptic effect not explained by elective excitation of nerve cells, and the experiment therefore becomes a test of the nature of at least certain types of reversal of inhibition.

It is proposed to illustrate this point by means of tests with platyhelminths, nemerteans, and annelids.

II.

Observations on the galvanotropism of common shore nemerteans were made in glass troughs containing sea water, through which a current of about 4 volts, 0.5 milliamperes per sq. cm., was sent by means of cotton pads. The current density was varied by changing the cross-section.

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[2] *Cf.* Moore, 1918–19, 1919–20; 1923–24, *a*. Crozier, 1919–20, 1922.

Several species of *Lineus* are pronouncedly cathodic. Most of the experiments were made with *Lineus socialis;* similar results were also obtained with *L. viridis*, a species of *Tetrastemma*, and another of *Cerebrotulus*.

In creeping the body is moderately elongated, and there is frequently no evidence of peristaltic waves; sometimes a succession of such waves may be apparent. When a current is sent through the water the nemertean undergoes marked elongation if the head is toward the cathode, and creeping continues in that direction. If the current is reversed, the body promptly shortens, and the anterior end, now toward the anode, becomes notably shortened and thickened; the worm may then move (backward) toward the cathode, but soon the anterior end extends to one side and both ends of the body are then directed to the cathode. Continued creeping brings the nemertean into cathodic alignment with the current. If at first the animal is transverse to the lines of current flow, both ends are at once directed to the cathode, the body becoming bent in a U. This behavior is essentially like that of the earthworm (Moore and Kellogg, 1916; Hyman and Bellamy, 1922; Moore, 1922–23, *b*) and is shown by other annelids, *Nereis* (Hyman and Bellamy, 1922) and *Harmothoaë*, as well as by marine and fresh water planarians. If the current be sent through the nemertean dorsoventrally, again the longitudinal muscles on the cathode side contract and produce the U-posture. As in the case of the earthworm (Moore, 1922–23, *b*), pieces or regions of the body exhibit similar responses. If a portion only of the intact nemertean be suspended in the current-trough certain differences appear. With the anterior end submerged and hanging vertically, closure of the current results in cathodic orientation and elongation of the submerged part; reversal of the current produces extensive shortening, so that the immersed part is lifted out of the water. Immersion of the posterior end only shows that part orienting with the posterior tip toward the anode, and reversal leads to contraction of the longitudinal muscles.

Isolated anterior ends of *Lineus* orient cathodically and elongate in this direction, shortening and turning on reversal. A transverse current produces the U-form. The behavior of isolated posterior parts is somewhat more complicated. Longitudinal muscles contract on the cathode side,—except occasionally just after cutting, when those on the anode side contract,—so that the typical U effect of a transverse current is produced; and the contraction of circular and dorsoventral muscles, with relaxation of longitudinals, leads to the usual elongation when the posterior end is toward the anode, which is changed to a shortening and thickening of the piece when the current is reversed. But peristaltic waves are commonly initiated at whichever end of the body happens to be directed toward the anode; and the piece creeps toward the anode whether the creeping be posteriorly or anteriorly directed; in a number of preparations it was clear that when in a U, with the two ends toward the cathode, peristaltic waves began at the anodic bend and moved, more or less alternately, to either end. Elongation of the fragment, however, occurs only when the posterior end is toward the anode, and reversal of the current produces the usual shortening and thickening; creeping with the anterior toward the anode is very slow, with faint, well spaced peristaltic waves and some wrinkling due to local circular muscle contraction. Occasionally, if antero-posterior peristalsis has been initiated it persists on reversal of the current, so that the piece creeps to the cathode. The behavior of pieces cut from the mid-region of the body, and thus with two cut surfaces, is similar. These, and the other isolated parts, were usually kept for about 24 hours before being tested.

The long proboscis of *Lineus* is not easily discharged.[3] Since the proboscis contains circular and longitudinal muscles it was desired to test its galvanotropism apart from the control of the nervous system in the body of the worm. The extracted proboscis shortens when its distal end is toward the cathode. Short fragments show longitudinal contraction on the cathode side. In thus paralleling the behavior of the whole body the proboscis is similar to the isolated tentacles of *Polyorchis* (Bancroft, 1904). It is an interesting question, however, as to the possibility of independent galvanotropic behavior of the parts of an animal. An illustration is afforded by the ceratia of eolids. *Æolida diversa* is cathodic, as are other nudibranchs which have been tested. *Æolida*, and *Dendronotus arboreacens*, elongate when the anterior end is toward the cathode; the tentacles and "rhinophores" bend to the cathode, but the ceratia (all but the most anterior four or six, in *Æolida*) bend to the anode. Reversal of the current causes retraction of oral and dorsal tentacles, shortening of the body, and the ceratia on the posterior part of the body stand out sharply and turn toward the anode. If the current is transverse, the animal shows extension of the ceratia only on the anode side. When autotomized posterior ceratia are studied it is found that they shorten when the distal tip is toward the cathode, elongate when the base is at the cathode; a transverse current produces longitudinal contraction on the anode side, so that both ends point to the anode. This effect cannot be due merely to the absence of the central ganglia because both anterior and posterior halves of transversely bisected individuals are found to creep and to orient cathodically. A further illustration of this sort of difference between the behavior of the body as a whole and of an appendage is found in *Echiurus*.

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[3] The proboscis of *Tetrastemma* was occasionally found to be everted when the anterior end was turned toward the anode, and retracted when this end completed its cathodic orientation.

The galvanotropism of *Echiurus chrysacanthophorus* was of especial interest for the experiments in view, because the orientation is anodic. A current sent transversely through the body leads to longitudinal contraction on the anode side, the body being thrown into a U. As the oral end turns to the anode, the circular muscles contract and this end elongates. Both normal and small regenerating proboscides, however, extend toward the cathode. With the body lengthwise in the current elongation is shown, particularly at the oral end, when the oral end is toward the anode; the proboscis is turned back toward the cathode. Peristaltic waves run from oral to aboral pole. When the current is reversed, the aboral end, now anodic, elongates markedly, and the oral end swings around so as to be directed to the anode. In some cases, locomotion being difficult on the smooth glass bottom of the trough, the animal progresses toward the cathode. Attention was chiefly given, however, to the interplay of circular and longitudinal muscles as the animal lay lengthwise to the current. Here it is obvious that longitudinal contraction is determined when the oral end is toward the cathode, longitudinal elongation and circular contraction when the oral end points to the anode. The isolated proboscis, however, shortens and thickens, its lateral edges and the distal end curling together, when the cathode is at the base of the organ; current reversal produces uncurling and longitudinal extension. Thus the proboscis may be considered intrinsically cathodic in its orientation, in contrast to the behavior of the body of the gephyrean.

The foregoing refers to the orientation of *Echiurus* lying on the bottom of a glass dish. At night, however, as described by Wilson (1899–1900), the worm can swim in an aquarium by means of vigorous peristaltic movements of the body wall; this performance is very striking in view of the mud-dwelling habit of the worm. When actively swimming *Echiurus* orients and swims to the cathode. Swimming movements were excited by repeated rapid reversals of the galvanic current, and orientation was then cathodic. Reversal of normally cathodic galvanotropism has been noticed in *Nereis* long confined to the laboratory (Hyman and Bellamy, 1922), but this is not the explanation of the behavior of *Echiurus*.

III.

The usual effect of strychnine upon flatworms and annelids is to produce (Knowlton and Moore, 1917; Moore, 1918–19) an enhanced excitability, and a reversal of the usual reaction to a local touch, for example, so that the contraction of longitudinal muscles is replaced by extension and the contraction of their antagonists. It is commonly observed that the gross effect of nicotine is to produce a shortening, thickening, or ventral flexure of the body of worms and other invertebrates (*cf.* Moore, 1919–20; Crozier, 1919–20; Crozier and Federighi, 1924–25). In the flatworms, nemerteans, and gephyrean used in the present experiments no unusual effects of strychnine or of nicotine were obtained, but it is necessary to record the results briefly, for the interpretation of the tests with the galvanic current.

Lineus in aquaria tends to collect in dark corners, but its negative phototropism is not pronounced except when first collected. The initial noticeable effect of treatment with strychnine sulfate (1:50,000) is the marked enhancement of this phototropism. Later a more or less stationary transverse enlargement of the body is apparent, at the level of the mouth; the lips are widely separated and the pharynx open. This is succeeded by the addition of more "standing waves", posterior to the mouth level; soon the whole body becomes much elongated. With higher concentrations the anterior half of the animal ultimately swells due to relaxation of the circular muscles as well as of the longitudinals, and the whole body is quite flabby. In solutions as concentrated as 1:2,000 the posterior region is very much attenuated, and the constriction of circular muscles pinches the body into fragments. Before this happens the body may be thrown into a coil, and in this the *dorsal* surface is always contracted, so that the animal may be said to be opisthotonic.

In the stage of excessive elongation, which with proper concentration of the drug lasts for some hours, creeping is entirely normal, but the reactions to tactile stimulation are reversed. A touch with a needle on the dorsal mid-surface of a normal *Lineus* leads to quick circular constriction at that level, coupled with longitudinal muscle contraction immediately anterior and then posterior; the two contractions of the longitudinal musculature run as waves in either direction; the circular constriction at the point touched persists for a time which varies with the severity of the excitation. If the animal be lifted from the bottom with a needle, it shortens, thickens, flattens; then the anterior end is extended and the circular constriction there begun progresses posteriorly.

A strychninized *Lineus* exhibits a stage in which the longitudinal shortening occurs anterior to the level of a touch, but the posterior part shows only a prompt lengthening. Then a little later it is seen that a gentle touch produces, not local circular constriction with attendant longitudinal contractions, but a local shortening *after* a local extension. The full development of this phase of the strychnine effect finds the animal responding to a touch by a violent contraction of the circular muscles, so that the whole body is enormously thinned and elongated. This reversal is also shown when the anterior end, with its ganglia, has been amputated, and indeed by short posterior pieces. At no time, however, does the animal spontaneously creep backward.

The two points which are of interest in the present connection are (1) the general elongation of the animal by strychnine, comparable to the galvanic effect with cathode at the anterior end, and (2) the reversal in the action of circular and longitudinal muscle groups following local stimulation.

There is one pertinent mode of response which does not seem to be affected by strychnine, at least until creeping is no longer possible. According to Moore (1923–24, *b*) the homostrophic reflex which tends to maintain the pursuit of a straight path in annelids (Morgulis, 1910; Moore, 1922–23) and in some arthropods (Crozier and Moore, 1922–23; Crozier, 1923–24) is not exhibited by *Cerebratulus*. The interest of this fact comes in connection with the suggestion (Moore, 1923–24, *b*) that the reflex excited by a lateral curvature of the body and which results in the anterior part being brought into alignment with the tail, might in some way be connected with segmental organization, since it is not seen in *Cerebratulus*, in planarians, or in the slug *Limax maximus*. But in a variety of the smaller nemerteans it is easily shown that precisely this homostrophic orientation of the anterior end is an important feature in directed creeping. Within a region extending back some 2 cm. from the anterior end typical homostrophic response is obtained in forward creeping, if the anterior end has been bent sharply to one side; slighter lateral displacements are effective nearer the anterior end. In backward creeping the posterior end continues to travel in the direction in which it is put, but the homostrophic adjustment of the anterior end is evident when this part of the animal travels backward through an impressed bend; the homostrophic orientation is therefore not dependent upon the direction of progression, but upon a central nervous state connected with the creeping act. "Beheaded" worms fail to show this behavior. The homostrophy of normal animals is accentuated by treatment with nicotine, but continues absent at other levels of the body.

IV.

When *Lineus* in the stage of strychninization characterized by elongation of the body and by reversal of the usual reaction to touch are subjected to the action of the galvanic current it is found that they *shorten* when the head is turned toward the cathode, *elongate* further if the anterior end is toward the anode, and that if placed transversely both ends of the body are turned to the anode. There is thus complete reversal of the usual galvanotropic behavior.

No reversal of galvanotropism could be secured by varying the current density. But at a slightly earlier stage in the strychnine action it is found that the longitudinal muscles on the cathode side of an animal transverse to the current will be contracted, as normally, but that when the anterior end is thus turned to the cathode the whole longitudinal musculature contracts and the worm shortens. To bring this about it is sufficient merely to bend the anterior end at the level of the mouth, so that the "head" is toward the cathode, the rest of the worm being parallel to the current lines, with posterior end to the cathode. Cases are found, also, in which the body is bent in a U with both ends toward the cathode, but with the longitudinal muscles contracted on the anterior half of the body.

These observations speak for the localization of the mechanism of strychnine reversal at the anterior end of the body. When the anterior part is amputated, at a level slightly posterior to the mouth, the worm shortens when the posterior end is at the cathode, elongates when the current is reversed. This proves that the strychnine reversal in the intact *Lineus* is due to an effect upon structures at the anterior end of the body, and it is natural to conclude that the cerebral ganglion is the essential locus. The isolated "heads" elongate with anterior tip toward anode, shorten on reversal. The "head" region so defined agrees with that evidenced by the homostrophic responses.

Isolated posterior halves are found to orient cathodically, although the end toward the cathode is broader than the posterior end, toward the anode. When the current is reversed the circular muscles at first contract, producing anodic elongation, but the anterior end turns toward the cathode.

V.

The galvanotropism of *Echiurus* supplies a complementary case. Treatment with strychnine (about 1:20,000, or less) causes the body to become more cylindrical, the circular muscles relatively more contracted; the proboscis is quite extended. Placed transversely to the current, both ends are bent toward the *cathode*, and the proboscis extended in this direction. Reversal of the current causes first the proboscis, then the oral end, then, less vigorously, the aboral end, to be swung toward the new cathode. In line with the current flow, the body extends when the oral end is cathodic, shortens when the oral end is anodic; in the first case the proboscis is extended, in the second bent back toward the cathode.

The galvanotropic behavior of the body muscles is therefore reversed by strychnine. But the conduct of the proboscis is unchanged. The isolated proboscis presents very much the appearance of a non-strychninized one, save that the distal end is kept more curled together and that its reactions are slower. Its behavior toward the galvanic current is not altered by the strychnine. The sort of independence of the central nervous system exemplified by the proboscis of *Echiurus* (and by the ceratia of *Æolida*) it is permissible to correlate with the fact that these organs are readily lost by autotomy.

VI.

These experiments began with the thought that the contraction of circular muscles, relaxation of longitudinals, characteristic both of the strychnine effect and of cathodal galvanotropism in *Lineus*, involve the activity of the same neuromuscular elements. The result showed that the typical effect of strychnine is not enhanced by the corresponding effect of the current, but that on the contrary the usual galvanotropic orientation suffers reversal under strychnine. The obvious conclusion is that whether or no strychnine "excites" by stimulation of nerve cell bodies, it must also, or perhaps primarily, have some other action which produces a true "reversal of inhibition"; and it is difficult to avoid the assumption that the locus of this reversal is in synapses (or their homologues). The results of removing the "brain" of *Lineus* strengthen this assumption, as does also the absence of the reversal in the proboscis of *Echiurus*. The fact that both the cathodal galvanotropism of *Lineus* and the anodal galvanotropism of *Echiurus* may be reversed by strychnine disposes of certain interpretations which might conceivably be put upon the behavior of one of these forms by itself.

A further test of this conception of the place of action of strychnine in effecting reversal of reaction is given by experiments with nicotine. Most of these experiments were made with several genera of marine platyhelminths. Exposure to dilute nicotine solution in time causes the longitudinal muscles of these worms to contract, the circular and transverse fibers to relax, so that the body is short and swoolen; the ventral longitudinal muscles are so contracted as to arch the body dorsally. If the galvanic current, presumed to act upon nerve cell bodies, should produce normally a similar effect, a common locus for the action of current and of nicotine would have to be assumed. Nicotine does not reverse the cathodic galvanotropism of planarians, nor of *Lineus*. But, in nicotine solution, even before the characteristic picture of nicotinization has begun to appear, the shortening, flattening, and arching of the body later produced by the alkaloid is clearly brought out during the flow of the galvanic current. The animals orient and move toward the cathode, but the body is broad and swollen, especially at the anode.

SUMMARY.

The cathodically galvanotropic orientation of nemerteans, *Lineus*, and the anodic orientation of the gephyrean *Echiurus*, are reversed by the action of strychnine under conditions such that the typical "reversal of inhibition" induced by this substance is apparent. Nicotine does not give this result. Since it is necessary to assume that the strychnine effect is due to action upon the central ganglia, and since the galvanotropic effect depends upon action of the current on nerve cell bodies of the central ganglia, it must be assumed that the locus of reversal by strychnine is not perikaryal, but presumably synaptic.

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PHOTOTROPISM IN YOUNG RATS.

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I.

A good deal has been written as to the limits of tropistic interpretation, particularly with regard to the behavior of higher animals such as hymenopterans and mammals. It seems to us that for any one who has seriously concerned himself with the analysis of animal conduct these discussions are for the most part extremely unsatisfactory. The study of the behavior of vertebrates has probably suffered a good deal from historically derived preoccupation with psychological questions. Perhaps the chief difficulty has been the absence of experiments likely to produce clear-cut and fruitful ideas. We are aware that to many the mere mention of "clear-cut ideas" in such connection is unpleasant. What we propose, however, is a system of analysis deliberately based upon a notion which to some would unquestionably appear a pernicious simplification. We have sought to discover well defined instances of tropistic conduct in mammals. These are of intrinsic interest. But their ultimate utility for our purpose consists in the fact that these tropisms, if found, may be brought into conflict, and the resolution of such conflicts studied under varied conditions (*cf.* Crozier, 1923–24, *a*, *b;* Crozier and Federighi, 1924–25, *a;* Crozier and Stier, 1926–27). It seems to us that the procedure of which this outline is a very bald statement should make possible a method of approach for the investigation of central nervous states otherwise inaccessible to measurement. It is a method, moreover, which frankly recognizes moment-to-moment variability in conduct, but which seeks to obtain quantitative evidence as to the nature of this variability and its physical basis.

It is necessary to find organisms capable of providing experimental results of the type required. For several reasons we have employed young rats and mice. Suitable tests have shown that they exhibit modes of behavior adapted for our purpose. And enough is known about them genetically to encourage the hope that it may be possible to utilize hereditary genes for the production of desired modifications of structural and other features.

The stereotropic behavior of these mammals has been described in a previous paper (Crozier and Pincus, 1926–27, *a*). We propose now to present proof of truly heliotropic conduct in young rats. It happens that the experiments seem not without significance for certain aspects of the general theory of phototropism. Beyond this, the occurrence of positive stereotropism and of negative phototropism during creeping makes it possible to bring these individually definite and predictable modes of response into conflict, in such a way that the animal must be guided by one form of response to the exclusion of the other, and to observe the manner in which the resolution of such conflicts may be modified. The nature of the results under these conditions will be discussed on another occasion.

II.

Conceived as a forced movement of the organism as a whole, tropistic conduct requires the presence of a preexisting mechanism, sometimes referred to as a "behavior pattern" (Child, 1924)—although this phrase may not in itself be particularly illuminating—so that the mode of response is a direct expression of the way in which the organism is constructed; and indeed there exist types of phototropic response, for example, which need not be interpreted in terms of adaptive requirements (Crozier and Arey, 1919–20). When the behavior of higher animals, such as man, is examined, attempts have been made to refer to the category of tropisms such phenomena as the guidance of movement by ideas (Royce, 1903) or by "memory images" (Loeb, 1918). Before analysis can make more than figurative headway in this direction it is necessary to discover if any simple and recognizable tropistic movements occur in mammals. The case of stereotropism has recently been discussed (Crozier and Pincus, 1926–27, *a*). The proof of geotropism is even more illuminating (Crozier and Pincus, 1926–27, *b*). To test for the presence of phototropism certain difficulties must be obviated. Complex image-forming or eidoscopic eyes, with pronounced central nervous connections, obscure potential tropistic response (Rådl, 1903; Parker, 1903; Parker, 1922, *b*). Thus the seeming positive phototropism of the young loggerhead turtle moving toward the sea (Hooker, 1911) is apparently the result merely of the retinal image of an unbroken horizon (Parker, 1922, *a*). Corresponding phenomena complicate the phototropism of imaginal insects (Rådl, 1906; Parker, 1903; Crozier and Federighi, 1924–25, *b*).

The eyes of nestling rats of the strain used do not open until the 14th day after birth. For some days before the eyelids open these animals respond to light. But during this time the eye must be regarded as a "direction eye" (euthyoscopic), not as an image-forming organ. Thus the opportunity is given to study the reactions induced by illumination, apart from the rôle of images, and apart from the effects of memory traces. The experiments may at once be controlled by opening the eyelids to discover the influence of retinal images of illuminated fields. The result shows that the closed lids provide a natural screen which precludes the formation of definite images, but which permits photic excitation.

Young rats of dark-eyed, black hooded stock were principally used in these experiments. The hooding factor produces a narrow band of dark hairs along the mid-dorsal line, which greatly facilitates the taking of graphical records of individual movements. While the eyelids are still closed the rats invariably move away from a source of light, with a promptness and precision rivalling that of a blow-fly larva. This is best seen between the ages 8 and 14 days; before the 8th day creeping is too uncertain to permit very definite orientation.

III.

One means of testing the phototropic nature of this response is to determine the character of the reaction when the animal is influenced by two sources of light. The lights used in these experiments were opposed at 180°. Young rats which start creeping on a line midway between two lights of equal intensity orient themselves in a direction perpendicular to the line joining the two lights (*cf.* Loeb, 1905; Patten, 1914). If the lights are of unequal intensity the path of oriented creeping is deflected toward the weaker light.

A black cloth, fastened to the observation table, permitted the animals to obtain a firm creeping grip. A system of circular and radial coordinates was marked in white upon the cloth, and allowed the changing position of the animal to be charted upon similarly marked record sheets. Creeping was begun near the center of the observation table, and the path was recorded until the outer marked circle had been reached. Only such complete trails were studied, the few instances being ignored in which, after very brief creeping, the animal squatted down on the cloth and refused to move. Each rat was "run" repeatedly, first with one side toward the stronger light, then the other. The intensities of the two lights were measured directly in the zone of creeping. Nitrogen-filled bulbs served as sources. The angle of orientation was measured from the charted record of each orientation test.

TABLE I.

Angles of orientation toward the weaker light, I_1 and I_2 opposed at 180°, H is the calculated effective inclination of the photoreceptive surfaces (see text).

I_1	I_2	θ	H/2
foot candles	*foot candles*		
8.0	8.0	0.19° ± 0.01	
8.0	7.0	5.98° ± 1.2	24.4°
8.6	7.0	15.8° ± 1.4	19.9°
8.6	5.7	22.4° ± 1.1	28.0°
8.6	4.6	27.9° ± 1.8	29.5°
8.6	3.9	32.2° ± 2.0	35.2°
8.2	3.4	31.1° ± 2.5	38.7°
9.5	3.1	41.0° ± 3.0	30.3°
10.3	2.9	44.3° ± 2.0	30.5°
12.0	2.67	47.4° ± 2.1	30.3°
14.0	2.65	52.0° ± 3.3	25.9°

The results of different series of experiments are collected in Table I. The consistency of the measurements of the angles of orientation, especially as seen in the small probable errors of the means, is surprisingly good.

Orientation by lights opposed at 180° should be definable (Crozier, 1926–27) by the equation

$$\tan \theta = \left(\frac{I_1 + I_2}{I_1 - I_2}\right)\left(\cot \frac{H}{2}\right) \qquad (1)$$

where θ is the angle between the line of orientation and the normal to the path of the light beams, I_1 and I_2 are the intensities of the lights ($I_1 > I_2$), and H is the average angle between the photoreceptive surfaces. It has been pointed out (Crozier, 1926–27) that this angle, H, should increase if orientation becomes more precise with increasing total acting light intensity; or, on the other hand, decrease if predominantly influenced by photokinetic movements; in either case H should be very nearly proportional to log $I_1 I_2$. In this way the variation of $H/2$ with log $I_1 I_2$ gives a test of the formula,—especially since cases are available in which H does not appreciably vary at all, and for which equation (1) holds with all desirable precision. For the rats, H decreases almost linearly as log $I_1 I_2$ increases (Crozier, 1926-27). An approximate graphical illustration of the relations between θ, I_1, and I_2 may be given (Fig. 1) by using the empirical relation

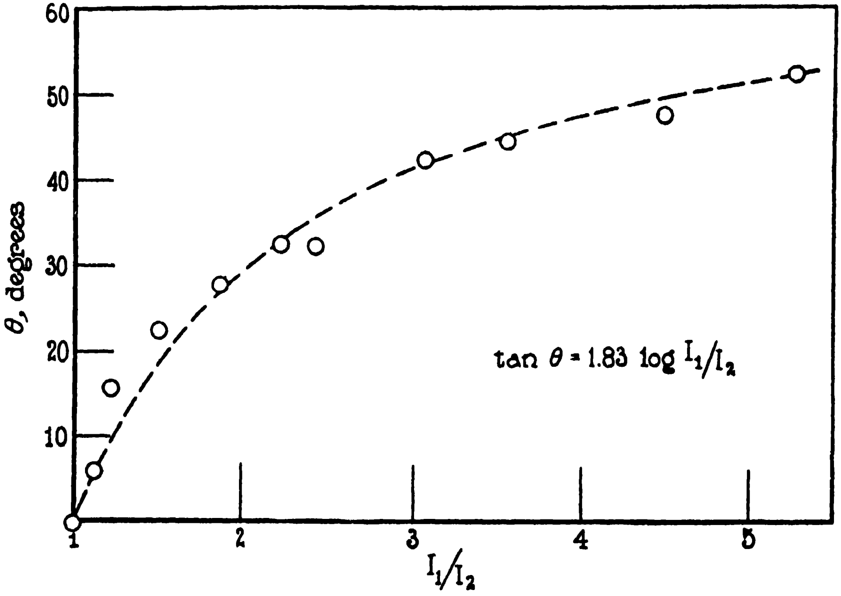

FIG. 1. The empirical formula tan $\theta = K$ log I_1/I_2 gives an approximate description of the variation in angle of orientation as dependent upon I_1 and I_2, and may be used in order to obtain a graphical representation of the results.

$$\tan \theta = K \log I_1/I_2 \tag{2}$$

obtained by M. M. Moore (1923–24) for such cases. It has been shown (Crozier, 1926–27) why, for ordinary ranges of intensities, this may give a very fair expression of the facts.

When I_1 and I_2 are equal, the path of orientation should be normal to the line joining the sources of illumination. The experimental finding of a mean deviation of only $-0.2° \pm 0.01$ is probably within the error of the method of recording. Beyond this, the way in which θ increases as the ratio I_1/I_2 is altered shows that the orientation fully obeys the primary requirement of phototropism, for the equation (1) connecting θ, I_1, and I_2 is based simply on the theory that orientation ceases when the illumination of bilaterally disposed photoreceptors is equal.

IV.

It has sometimes been held that the results of experiments with opposed sources of light mean merely that the organism "seeks the dark". With the rat there is a rather neat way of removing this opinion, although it is obvious that the results contained in Table I should be sufficient demolition of it.

When the eyes open, a different situation results. In general, the young rats then seek the darkest region in the field of view, usually the shadows on either side of the lamp house. Older animals face the light, and creep toward the darkness at one side of it. If a young rat is placed facing the light, and at a point midway between two symmetrical zones of shade, it moves *away* from the light, in a straight line. If, however, it be placed nearer one shadow than the other it moves at once to the nearer, even moving almost directly *toward* the light to do so. If put at some distance from a lamp (say, 6 feet from a 100 watt bulb), it usually turns away from the light toward the recesses of the dark room. There seems to be a zone in which the tendency to turn away from the light and the stimulus leading to creeping toward the dark corners at the sides of the lamp house are about equalized. Thus even with the eyes opened there is still some evidence of negative phototropism, but it is clear that there is superimposed upon its simpler manifestation the positively orienting influence of darkened areas contrasting with a brighter one in the visual field (*cf.* also the results of learning tests with rats; Yerkes, 1907; Watson, 1914).

The type of response determined by the visual effect of darkness is obtained in rats kept in the ordinary illumination of the laboratory, or in those continually maintained in darkness until after the eyes have opened. It is secured at once if the eyelids are opened by operation before their normal time. This makes it possible to show directly that if the animal were "seeking the dark", in the sense in which this expression may be used with reference to vision, it would scarcely move in the way recorded in Table I.

V.

Another way of testing the occurrence of phototropism has to do with the examination of circus movements. With the young rats there are three possible ways of doing this. (*a*) One eye may be opened, at the age of, say, 12 days, the other eye remaining closed; or (*b*) one of the two unopened eyes may be covered by an opaque screen; or (*c*) one eye may be extracted.

In the first case, (*a*), care must be taken to prevent the possibility of image formation in the opened eye, by presenting a uniform visual field. When this is done it appears that the primitive phototropic effect, already noted as persisting in connection with the recently opened eye, brings it about that the rat circles slightly *toward the unopened eye.* This would result if stimulation of the opened eye were merely more intense, which is pretty certainly the case. These tests were made by illuminating prepared rats by a ring of electric bulbs suspended from a large circular metal frame, within which the rat crept. The lamps were brought within 3 feet of the creeping surface. This method is necessary to avoid shading of one eye by the rat's head, as when a single point source is used. With *high* total intensities of light (1000 m.c.) no definite circling to either side is obtainable.

The second method, (*b*), has disadvantages, because it is difficult to attach an adequate screen which the animal will ignore and which is small enough not to interfere with the vibrissæ.

The third procedure, (*c*), results in very clear-cut exhibitions of maintained circling toward the blind side. The amount of turning depends, as in *Limax* (Crozier and Federighi, 1924, *a*) upon the speed of creeping. With high intensities of illumination the rat squats more or less at one point and pivots about the strongly flexed hind limb of

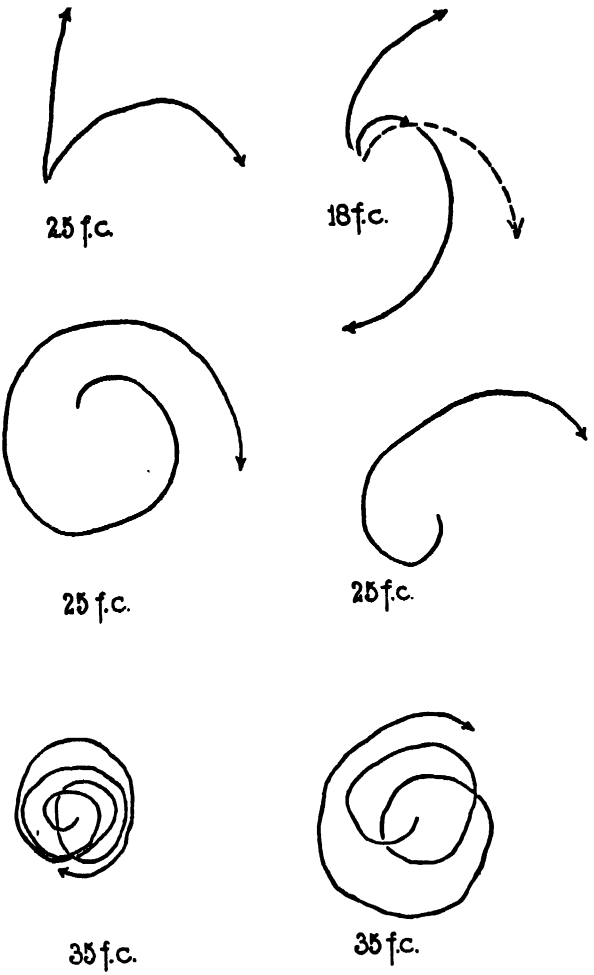

FIG. 2. Circus movement trails of young rats with one eye (the right) removed, the eyelids of the other not yet parted. Illumination from a single source, vertically above the center of the creeping stage. Intensities of the lights (foot candles) measured photometrically at the creeping stage. Scale 1:10. These trails are typical. The amplitude of the orientation movement increases with the intensity of the light. This is in part due to the fact that the animal creeps little but turns constantly. The records were obtained by following the positions of a marked spot at shoulder level on the animal's back, as seen against a system of coordinates in white lines upon a black cloth covering the creeping stage.

the blind side. More creeping movement is evident with lower intensities of light. Several typical trails of circling progression are given in Fig. 2, and fairly illustrate the type of behavior constantly observed. The postures of the legs are strikingly influenced by the light. Extensor tonus is enhanced on the photoreceptive side, and most markedly in the anterior limb; the legs of the other, blind, side are flexed, the hind leg much more so. The turning movement is brought about through the pushing effect of the extended legs, coupled with protraction by those of the blind side. The tonic effect of the light (Fig. 3) is as definite here as with insects (Garrey, 1918–19; Crozier and Federighi, 1924–25, *a*). Under continuous exposure the circle of turning broadens into a spiral, due to light adaptation. We may point out that this gives promise of providing a method for the quantitative treatment of light adaptation.

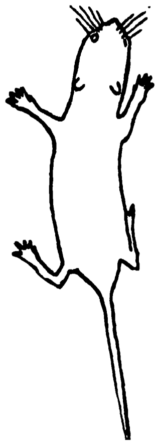

FIG. 3. The orienting posture of a rat, left eye functional but as yet unopened, right eye removed, illuminated from above.

VI.

The result of these tests is to show that the quantitative criteria supplied (1) by the action of opposed illuminations and (2) by the requirement of circus movements, agree in demonstrating the elementary tropistic nature of the movements of the rat as influenced by light. Upon this basic phototropism the image-forming capacity of the opened eye imposes certain modifications and restrictions. If one desires to employ quantitatively predictable behavior as an index instrument for analysis of conduct, it is necessary to choose experimental conditions such as permit the organism to display its capacities as a machine. The phototropism of the rat may thus be studied if the effect of visual images is avoided; and if young rats are employed, before the opening of the eyelids, there is not even a possibility of the memory of such images.

VII.

SUMMARY.

Before the eyelids have opened young rats are negatively heliotropic. They behave very much as does the larva of the blow-fly. The angle of orientation by lights opposed at 180° may be calculated by an equation based upon the elementary requirement of phototropism, namely that orientation is attained when the illumination of bilaterally disposed photoreceptors is equal. The precision of orientation decreases very nearly in proportion to the sum of the logarithms of the acting light intensities, due to photokinetic head movements. When the eyelids are opened, the rats move toward a darkened place in the field of vision, usually toward the shaded region immediately to one side of the lamp house. Therefore, when heliotropic, the rat is not "seeking the dark". The phototropism of these animals may be brought into conflict with their pronounced stereotropism, and the resolution of such conflicts may perhaps be utilized for the investigation of central nervous states.

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ON THE EQUILIBRATION OF GEOTROPIC AND PHOTOTROPIC EXCITATIONS IN THE RAT.

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I.

Analysis of the phototropic movements of young rats (Crozier, 1926–27; Crozier and Pincus, 1926, 1926–27, *b*) has justified the assumption that in the absence of image formation, insured by employing animals with eyelids still unopened, these mammals behave as phototropic machines, with no detectable intervention of a central nervous factor.[1] The young rat, in a field of light, orients until the excitations on its two sides are equal; over the range of intensities used, excitation is proportional to the logarithm of the intensity of the light. Geotropic orientation of these animals, also, is proportional to the logarithm of the active gravitational pull, although the reason for this particular relationship (Crozier and Pincus, 1926, 1926–27, *a;* Pincus, 1926–27) is probably not the same as in the case of phototropic excitation.

These investigations began with the double objective of discovering the limits of tropistic analysis as it might be applied to the conduct of mammals, and of utilizing the results for the study of central functions (Crozier, 1926–27). With reference to the latter purpose it may be pointed out that there are at least two general methods possible to pursue. One of these consists in the opposition of tropistic modes of response in such ways as to permit quantitative evaluation of the resolution or equilibration of the imposed conflicts in behavior (Crozier, 1926–27; Crozier and Pincus, 1926). The conduct of the young rats in a compound field of excitation involving opposed phototropic and geotropic responses provides an elementary situation amenable to precisely this sort of treatment.

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[1] Aside from that involved in photokinetic movements of the head, which is itself amenable to quantitative treatment in the same terms (Crozier, 1926–27).

The simplicity of the actual result gives the best possible proof of the adequacy of the initial assumptions with regard to the mathematical representation of the phototropic and the geotropic excitations. It also constitutes a second-order proof that in the resolution of such conflicts the rat is behaving as a machine. There are several ways in which phototropic stimulation might be conceived to modify the effectiveness of simultaneous geotropic excitation: (*a*) the constants in the equation for the geotropic effect might be modified, or its threshold altered; or (*b*) the whole form of the equation might be changed, either at once or as a function of the time of exposure to the light. The possibility first mentioned (*a*) could be dealt with very simply, and obviously could give a means for the quantitative expression such as we seek for a "central factor." The second possibility might be expected if complex psychic processes are implicated, but change with time cannot be simply tested for, because of photic adaptation. The net result of these considerations is, that if from the respective known expressions for the phototropic and the geotropic effects it is possible to predict the mathematical form of the balance between phototropic and geotropic excitations there will remain no need nor indeed any room for a psyche in this particular case. Since this is the actual result, it will be noticed that the initial assumptions of the tropism doctrine are doubly confirmed. So far as we are aware this is the first case in which just this sort of analysis has been possible, and the fact that it has been carried out with a mammal makes it all the more interesting.

II.

The rats used in previous analysis of phototropism and of geotropism were of the same genetic history as those employed for the present experiments, and in this respect the results are strictly comparable. The phototropic conduct has been treated as depending upon the fact that the excitation is proportional to log I. The geotropic response upon an inclined plane is such that the angle of upward orientation on this plane is proportional to log sin α, where α is the inclination of the creeping plane to the horizontal. If the arrangement be made such that the intensity of light is found at each of a number of values of α which is just sufficient to suppress geotropic orientation, we should expect that

$$\log I = K \log \sin \alpha + C,$$

where K and C are constants. This could be the result only if the presence of the light failed to affect the form of the connection between geotropic excitation and response. The actual result should therefore afford a criterion for the evaluation of the kind of central nervous adjustment eventuating from the competition of the two forms of stimulation. The significance of the constant C cannot be directly determined, for we do not yet know the magnitude of the corresponding quantity in the equation for phototropic effect; but, as we shall point out subsequently, it may be estimated indirectly.

Two series of experiments were made according to the following procedure. The rats were dark-eyed, black hooded, 13 days after birth. In Series 1, three individuals were employed; in Series 2, 4,—litter mates in each case. Tests were made upon a fine-meshed wire screen stretched upon a large square platform which could be inclined at known angles to the horizontal. This creeping stage was at one end of an extended arm carrying the light source, the latter adjustable as to distance from the stage. The dark-adapted rats were placed upon the stage, one at a time, and oriented upward toward the light until the light intensity became such that the animal was forced to creep in a horizontal path. It may be assumed that at this point the upward orienting tendency is exactly counteracted. The intensities were measured photometrically, not computed. The total exposure to the light was kept at 1 minute, with at least 10 minutes in darkness between successive tests. By repeated trials the exact distance was found at which horizontal creeping was enforced; the tests were repeated five times at each inclination, for each individual.

III.

The results of the first series of tests, when $\log I$ was plotted against $\log \sin \alpha$, gave I proportional to $(\sin \alpha)^K$, with $K = 2.475$. The second series of measurements, treated separately, gave $K = 2.476$.

This degree of concordance is probably fortuitous, but we would stress the point that the individuals concerned in the two sets of determinations were unusually similar from the standpoint of age and genetic uniformity. The two series of measurements are plotted together in Fig. 1, where it is obvious that they form a homogeneous whole.

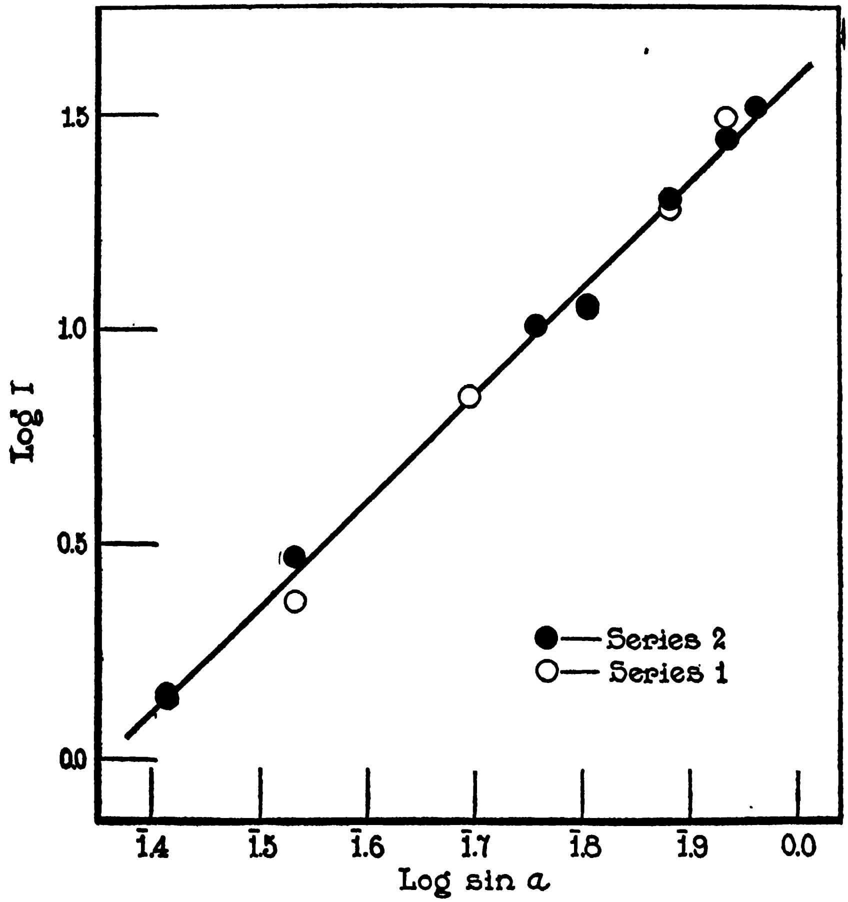

FIG. 1. The intensity of light required to inhibit geotropic orientation of young rats varies with the inclination of the creeping surface, in such a way that log I/log sin α is constant. For Series 1 each point is the mean of fifteen determinations; in Series 2, of twenty determinations.

Inasmuch as the variability of the response to the geotropic stimulus decreases in proportion to log sin α (Crozier and Pincus, 1926–27, *a*), and since the photokinetic sideward movements of the head increase in proportion to log I (Crozier, 1926–27; Crozier and Pincus, 1926–27, *b*), it is to be expected that the two recognizable sources of variation in the apparent equilibrating light intensity must probably tend to counterbalance. The variability of the intensity required to equal the geotropic stimulation should therefore be fairly small. To test this, the probable errors of I have been computed (Table I); expressed as percentages of the mean intensities, which give figures corresponding to the coefficient of variation, it is seen that in agreement with this expectation the variability of I as measured is quite low. The quantity which inversely measures the photokinetic effect (Crozier, 1926–27) decreases linearly as log I increases, while the variability of geotropic orientation directly decreases as log sin α increases (Crozier and Pincus, 1926–27, *a*). Thus it is to be expected that the coefficient of variation for I in the present experiments should pass through a minimum as α increases from 10° to 70°. The differences among the probable errors in Table I are statistically significant. Fig. 2 shows that the variability of I, as α is increased, does pass through a minimum in the expected way.

TABLE I.

Intensities of Light Required to Counterbalance Geotropic Excitation at Different Angles of Inclination (α) *of the Creeping Plane.*

	α	Log sin α	I, mean	Log I	P.E. of I, as per cent of mean
			foot candles		*per cent*
Series 1	15°	$\bar{1}.413$	1.413	0.150	0.47
	20°	$\bar{1}.534$	2.353	0.372	0.38
	30°	$\bar{1}.699$	7.040	0.848	0.26
	40°	$\bar{1}.808$	11.22	1.050	0.26
	50°	$\bar{1}.884$	19.43	1.288	0.15
	60°	$\bar{1}.938$	31.19	1.493	0.77
Series 2	15°	$\bar{1}.413$	1.387	0.1421	0.35
	20°	$\bar{1}.534$	2.968	0.4725	0.79
	35°	$\bar{1}.759$	10.19	1.0082	0.44
	40°	$\bar{1}.808$	11.45	1.0588	0.49
	50°	$\bar{1}.884$	20.04	1.3019	0.41
	60°	$\bar{1}.938$	28.08	1.4484	0.97
	70°	$\bar{1}.973$	33.43	1.5241	0.83

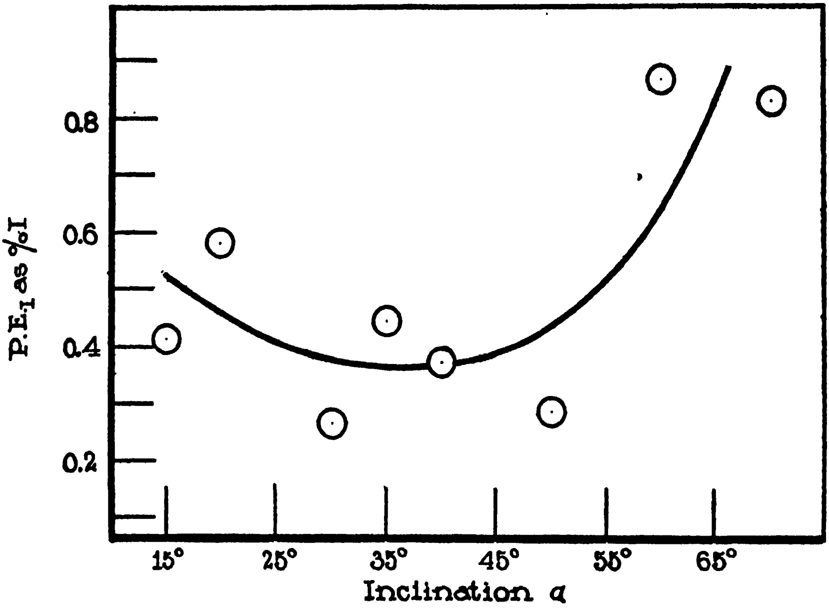

FIG. 2. The variability of the intensity required to counterbalance geotropic excitation passes through a minimum as the angle of inclination of the creeping surface is increased (see text).

IV.

SUMMARY.

The intensity of light required to just counterbalance geotropic orientation of young rats, with eyelids unopened, is so related to the angle of inclination (α) of the creeping plane that the ratio log I/log sin α is constant. This relationship, and the statistical variability of I as measured at each value of α, may be deduced from the known phototropic and the geotropic conduct as studied separately, and affords proof that in the compounding of the two kinds of excitation the rat is behaving as a machine.

CITATIONS.

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CERTAIN EFFECTS OF SALTS ON THE PENETRATION OF BRILLIANT CRESYL BLUE INTO NITELLA.

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I.

INTRODUCTION.

The effect of salts on the rate of penetration of dye into living cells has been studied by various investigators[1] by comparing the rate in dye solution containing salt with that in dye solution containing no salt, but it is not possible to determine from their results whether the salt acts on the protoplasm or on the dye or on both.

The writer therefore has suggested a method[2,3] by which we are able to separate the effect of salt on the protoplasm from that on the dye. This is done as follows: (1) Cells are placed for a given time in a salt solution, after which they are placed in dye solution containing no salt. The rate of penetration of dye in the case of such cells is compared with that of the control (cells which have not been previously exposed to the salt solution). This gives the effect of salt on the protoplasm since it has previously been shown[2,3] that there is no error due to salt adhering to the surface of the cell wall (which cannot be removed by wiping and washing). It might be thought that an error could arise from the fact that salt might diffuse out from the cell when removed from the salt solution and placed in the dye solution containing no salt, and enough of this might collect in the solution just outside the protoplasm to affect the results. This, however, has been shown[2,3] not to take place. (2) Cells previously exposed to water or salt solution are placed in dye solution containing the salt, and the rate of penetration in these cells is compared with that of the control (cells previously exposed to distilled water and then placed in dye solution containing no salt).

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[1] *Cf.* Endler, J., *Biochem. Z.*, 1912, xlii, 440; xlv, 359. Szücs, J., *Sitzengsber. k. Akad. Wissensch., math.-naturw. Cl., Wien*, 1910, cxix, 1. These papers contain references to earlier literature. Lack of space prevents a detailed comparison of their results with those of the writer, but it may be said that in general they found that in some cases the presence of salts in the dye solution accelerated and in other cases inhibited the penetration of dye.

[2] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 54.

[3] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 271.

Preliminary accounts of such experiments have been given in the writer's previous papers.[2,3] These experiments are of interest because they are helpful in locating the factor controlling the rate of penetration of dye under various experimental conditions, which is one of the important problems in permeability.

The theory underlying the mechanism of the penetration of basic dye into living cells has been presented[4] by the writer and need not be discussed in detail here. But it may be stated that we assume that we are concerned only with the diffusion of the dye in the form of free base (for convenience called DB) into the vacuole, since the salt (called DS for convenience) does not enter the cell very readily. As DB enters[5] the vacuole, some of it changes to DS, and more DB enters until there is in the vacuole a definite ratio of DB/DS depending on the condition of the sap, *e.g.* the pH value and the salt content. At equilibrium, DB[6] in the vacuole is either equal to or proportional to DB in the external solution, depending on the solubility, etc.

II.

Methods.

Since the method of determining the amount of dye in the vacuole and the general experimental precautions and procedure have been repeatedly described in the writer's previous publications, a detailed account will not be given here.

The determination of the amount of dye in the sap was made colorimetrically. The dye, brilliant cresyl blue, was dissolved in M/150 borate buffer at pH 7.7 unless otherwise stated. The pH values of the solutions were determined by means of the hydrogen electrode. The experiments were carried out in an incubator at 25 ± 0.5°C.

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[4] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 75.

[5] The same may be said of DB in the protoplasm.

[6] What we actually measure is the total dye (DB and DS) but since the color of DB and DS is the same, and since there is a definite ratio of DB/DS, we are justified in using this method.

Only living cells (collected in Cambridge unless otherwise stated) free from foreign growths, such as diatoms on the surface, were used and great care was taken to obtain uniform cells and to keep them all under the same conditions before experimentation. The cells were invariably wiped and washed in distilled water for 5 seconds, and again wiped, before they were placed in any solution.

Each experiment represents an average of about 60 readings on individual cells, and the probable error of the mean is less than 7 per cent of the mean.

In carrying out a series of comparative experiments, approximately the same number of readings was made for each experiment each day, and an average was taken of all the readings of each experiment after 60 readings were made. There may be a great source of error if comparative experiments are not made at the same time and with uniform cells.

III.

Salts with Monovalent Cations.

The experiments[7] (see Table I) with 0.013 M NaCl solution show that the rate of penetration of dye (as compared with that of control cells transferred from distilled water to the dye solution) decreases greatly when cells, after exposure to the salt solution for 10 minutes,

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[7] The experiments were carried out as follows: Cells were divided into four groups:

(*a*) Cells were placed for 10 minutes in distilled water (pH 5.4), after which they were wiped and placed in 0.00014 M brilliant cresyl blue solution at pH 7.7 (M/150 borate buffer mixture). After 2 minutes the concentration of the dye in the sap was determined. This experiment represents the control, and this rate of penetration is used as a standard of comparison.

(*b*) Cells were exposed for 10 minutes in 0.013 M NaCl solution (made up in the distilled water), after which they were wiped and washed for 5 seconds in distilled water. Then they were again wiped and placed in the same dye solution as group *a*. After 2 minutes the concentration of dye in the sap was determined.

(*c*) Cells were placed in distilled water for 10 minutes, after which they were wiped, and placed in 0.00014 M dye solution containing 0.013 M NaCl at pH 7.7 (M/150 borate buffer mixture). After 2 minutes the concentration of dye in the sap was determined.

(*d*) Cells were exposed for 10 minutes in 0.013 M NaCl solution, after which they were wiped, and washed for 5 seconds in distilled water. Then they were again wiped and placed in the same dye solution as group *c*. After 2 minutes the concentration of dye in the sap was determined.

TABLE I.

Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of Nitella in Presence of NaCl at Different Concentrations.

I. Concentration of external NaCl solution.

II. Concentration of dye in the vacuole when cells were placed in NaCl solution (Column I) for 10 minutes and then placed in 0.00014 M dye solution at pH 7.7 (borate).

III. Concentration of dye in the vacuole, when cells were placed in distilled water for 10 minutes and then in 0.00014 M dye solution at pH 7.7 (borate) containing the same concentration of salt as in I.

IV. Cells were placed for 10 minutes in the salt solution stated in I, and then placed in the same dye solution as in III.

As a basis of comparison the concentration of dye in the vacuole was taken when cells were placed in distilled water for 10 minutes and then placed in the same dye solution as in II. This concentration was 25.0×10^{-5} M.

In all cases the concentration of dye in the vacuole was determined after 2 minutes exposure to the dye solution.

I	Concentration of dye in the vacuole		
	II	III	IV
M	$M \times 10^5$	$M \times 10^5$	$M \times 10^5$
0.0500	7.9	30.1	33.0
0.0250	7.2	29.3	32.2
0.0125	6.9	29.5	31.4
0.0063	7.6	29.3	30.5
0.0016	15.5	23.8	23.7
0.00063	22.8	24.5	24.0

TABLE II.

Concentration of brilliant cresyl blue in the vacuole of living cells of *Nitella* when cells previously exposed to tap water or for different lengths of time to distilled water or to 0.05 M or 0.01 M NaCl solution are placed in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) for 2 minutes.

In tap water at pH 7.0	In distilled water pH 5.4		In NaCl solution				
	1/2 min.	10 min.	5 sec.	1/2 min.	2 min.	5 min.	10 min.
$M \times 10^5$	$M \times 10^5$	$M \times 10^5$	$M \times 10^5$	$M \times 10^5$	$M \times 10^5$	$M \times 10^5$	$M \times 10^5$
22.4	21.4	22.1	24.2	5.2	5.9	4.5	5.2

are subsequently placed in the 0.00014 M dye solution made up with borate buffer mixture at pH 7.7 for 2 minutes. Cells previously exposed either to distilled water or to 0.013 M NaCl for 10 minutes and then placed for 2 minutes in the same concentration of dye, at the same pH value, containing 0.013 M NaCl solution, show a slight increase in the rate of penetration of dye (Table I).

These experiments were repeated with different concentrations of salt between 0.05 M and 0.0006 M (Table I). Both the inhibiting and accelerating effects of NaCl remain about the same between 0.05 M and 0.006 M, but below the latter concentration both effects diminish as the concentration decreases until at 0.0006 M solution there is practically no more effect.

TABLE III.

Experiments Showing That the Effect of NaCl on the Protoplasm Causing a Decrease in the Rate of Penetration of Brilliant Cresyl Blue into the Vacuole of Nitella (Collected in New York) Is Not Readily Reversible.

I. Represents the control experiment. Cells placed for 10 minutes in distilled water and then placed in the dye solution.

II. Cells were exposed for 2 minutes to 0.0016 M NaCl solution after which they were placed in the dye solution.

III. Cells were exposed for 2 minutes to 0.0016 M NaCl after which they were washed in a large volume of distilled water for 1 hour, and then placed in the dye solution.

In every case the cells were placed for 1 minute in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture).

	I	II	III
	M × 10^5	M × 10^5	M × 10^6
Amount of dye in sap	31.8	18.9	20.0

This inhibiting effect of NaCl may be brought about in ½ minute at 0.01 M or above (Table II). This effect is not removed by transferring the cells after 2 minutes from the salt solution to distilled water and leaving them for 1 hour, as shown in Table III.

When the experiments were repeated with KCl, KNO_3, LiCl, Na citrate, and Na_2SO_4 at 0.01 M solution the same results were obtained as is shown in Table IV.

TABLE IV.

Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of Nitella in the Presence of Salts.

I. Cells were exposed for 10 minutes to 0.01 M salt solution after which they were placed in the 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture).

II. Cells were exposed for 10 minutes to distilled water after which they were placed in the 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) containing 0.01 M salt.

III. Cells were exposed for 10 minutes to 0.01 M salt solution after which they were placed in the same dye solution as in II.

As a basis of comparison the concentration of dye in the vacuole was taken when the cells were placed in distilled water for 10 minutes and then transferred to the same dye solution as in I. This concentration was 24.2×10^{-5}M.

In all cases the cells were placed in the dye solution for 2 minutes.

0.01 M external salt solution.	Concentration of dye in the vacuole		
	I	II	III
NaCl	6.9	28.1	31.0
LiCl	7.9	27.8	32.5
KCl	8.3	29.0	31.3
KNO_3	7.3	27.5	30.0
Na_2SO_4	5.8	29.5	32.2
Na citrate	5.5		
$CaCl_2$	26.0	26.2	26.5
$MgCl_2$	26.2	25.9	27.0
$MgSO_4$	25.8	26.5	26.8
$LaCl_3$	29.2		
$La(NO_3)_3$	28.3		

IV.

Salts with Bivalent and Trivalent Cations.

When the experiments[8] were carried out using salts with bivalent and trivalent cations, namely,[9] $MgCl_2$, $CaCl_2$, $MgSO_4$, $LaCl_3$, and

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[8] These experiments were carried out by dividing the cells into four groups in the same manner as with NaCl, as described in Foot-note 7, which should be consulted for the details of the experiments.

[9] $LaCl_3$ and $La(NO_3)_3$ reduced the pH value of the solution considerably so that the experiments were carried out with lower concentrations of the salt and the same result was obtained.

$La(NO_3)_3$, it was found that there is a very slight accelerating effect in the case of cells previously exposed to the salt solution and then placed in the dye solution containing either salt or no salt, as well as in the case of cells directly transferred from distilled water to the dye solution containing salt (Table IV).

TABLE V.

Experiments Showing That the Effect of NaCl on the Protoplasm Causing a Decrease in the Rate of Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of (New York) Nitella May Be Removed by Salts with Bivalent and Trivalent Cations.

The cells were left for 5 minutes in each salt solution, and then placed in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) for 1 minute.

The cells were exposed to the various solutions in the order given. The cells kept in distilled water for 5 minutes and then transferred to the dye solution for 1 minute had 28×10^{-5} M dye in the vacuole.

The cells exposed to 0.001 M NaCl for 5 minutes and then transferred to the dye solution had 15.6×10^{-5} M dye in the vacuole.

Salt solution	0.001 M NaCl, then salt solution, then dye	Salt solution, then 0.001 M NaCl, then dye
	M $\times 10^5$	M $\times 10^5$
0.01 M $MgCl_2$	29.5	16.1
0.01 M $MgSO_4$	30.0	16.6
0.001 M $MgSO_4$	29.2	15.0
0.001 M $LaCl_3$	28.4	17.0

In considering the results obtained with all the salts given above, we may conclude that the decreasing effect[10] of the salts with monovalent cations is due to the effect of the cations and not to that of the anions, while the bivalent and trivalent cations have no inhibiting effect on the protoplasm.

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[10] For results with certain concentrations of dye and salts (including a decrease in penetration of dye in dye solution containing either 0.05 M $CaCl_2$ or $MgCl_2$ at higher pH value). See Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, No. 4.

V.

The Inhibiting Effect of NaCl May Be Removed by Salts with Bivalent and Trivalent Cations.

The question now arises whether the inhibiting effect of NaCl may be entirely or partially removed by other salts. To test this question the following experiments were made.

Cells were exposed to 0.001 M NaCl solution for 5 minutes after which they were washed in 0.01 M $MgCl_2$ for 5 minutes, and then transferred to the dye solution. The inhibiting effect of NaCl was found to have been completely removed[11] since the rate of penetration of dye was the same as that of the control, as shown in Table V. This inhibiting effect, however, was not removed when cells were first exposed to the $MgCl_2$ solution and subsequently treated with NaCl and then placed in the dye solution (Table V)

The same result is obtained (Table V) when $LaCl_3$ and $MgSO_4$ are used instead of $MgCl_2$.

No inhibiting effect of NaCl is produced if cells are exposed to 0.02 M NaCl containing 0.01 M $MgCl_2$ and then placed in the dye solution. In some cases the inhibiting effect of NaCl is removed in dye made up with phosphate buffer mixture, or with borate buffer mixture at high pH value in which borax predominates, or at lower pH value containing NaCl or $MgCl_2$.

Theoretical Considerations.

The experimental results described above appear to involve two factors, (1) effect of salt on the dye, (2) action of salt on the protoplasm (either at the surface or in the interior).

A salt (with monovalent, bivalent, or trivalent cations) may affect the dye in three ways, (*a*) increase the dissociation of the dye, (*b*) decrease the solubility of the dye, (*c*) have some other (possibly specific) effect on the dye.

In order to study the effect of salt on the dye experiments were made on the distribution of the dye between chloroform and the 0.00014 M dye solution (borate buffer mixture) at pH 7.7. These show that the addition of 0.01 M NaCl or 0.01 M $MgCl_2$ to the dye solution does not increase the amount of dye taken up by the chloroform as would be the case if the dye were being salted out but on the contrary decreases it, which may indicate that the solubility of the dye (at such concentrations of the dye and the salts as are here employed) is not affected as much as the dissociation constant of the dye. The salt appears to increase the dissociation of the dye which would cause less dye to enter the chloroform.

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[11] The inhibiting effect brought about by 0.01 M NaCl may also be removed by phosphate buffer mixture containing 0.00014 M dye, while it cannot be removed by the buffer solution containing no dye.

The question arises whether we can apply these results directly to *Nitella*. In view of the fact that the rate of penetration of the dye at various pH values was previously found[4] by the writer to correspond with the amount of dye absorbed by the chloroform from aqueous solution at these pH values, we might expect a similar correspondence to exist when cells are placed in the dye solution containing the salt, but the experiments show the contrary. Less dye is taken up by the chloroform while more dye is taken up by the cells when the aqueous dye solution contains salt. This may indicate that the effect of change in solubility exceeds that of the shift in dissociation constant, in the case of *Nitella*, since the partition coefficient of DB between the cell vacuole and the external solution is small, probably less than 1. Hence a slight change in the solubility of DB in the external solution may bring about a great change in the partition coefficient, while the partition of DB between chloroform and M/150 buffer solution is found to be exceedingly large so that a slight change in the solubility may have no appreciable effect on the partition coefficient. But it does not seem possibe to explain these results on *Nitella* entirely on the basis of solubility because the salting out effect of a salt with monovalent cation, such as NaCl, would probably be less than that of a salt with bivalent cation, such as $MgCl_2$, so that at the same concentrations one might expect less accelerating effect with NaCl than with $MgCl_2$, but the experiments show that NaCl brings about a greater effect than $MgCl_2$ (which has very little effect) which indicates that the situation may be complicated by additional factors such as the specific effect of cations on the dye and on the protoplasm.

That the accelerating effect in dye plus NaCl is due to the direct action of the salt on the dye in the external solution and not on the protoplasm is shown by the experiment where the cells are exposed to the salt solution and then placed in the dye solution containing no salt, in which case there is a decrease in the rate of penetration of dye instead of an increase. This decrease cannot be due to the entrance of salt into the vacuole since the halide content remains unchanged. It must therefore be due to the presence of the salt in the protoplasm (either at the surface or in the interior). We may then assume that the salt acts on the protoplasm in such a way that the rate of penetration of dye from the external solution into the protoplasm is decreased (it might, for example, decrease the solubility of dye in the protoplasm or increase the viscosity of the protoplasm) and if the penetration of dye into the outer surface of the protoplasm is slower than the diffusion of the dye from the protoplasm into the vacuole we may expect a decrease in the amount of dye in the vacuole.

A striking fact is that this inhibiting effect not only disappears but is replaced by an accelerating effect when cells thus exposed to the salt solution are placed in the dye solution containing salt. The accelerating effect is a little greater in this case than when cells are first exposed to distilled water and then placed in the same dye solution. It has been shown by experiments that at the concentration of NaCl employed the effect of the salt on the protoplasm is immediate so that irrespective of whether the cells have been formerly exposed to the salt or not the condition of the protoplasm may be practically the same very soon after the cells are placed in the dye solution containing the salt.

If this is true we may assume that the reason the accelerating effect is a little greater in the case of the cells previously exposed to the salt is because there is a greater accumulation of salt in the very thin film of liquid between the protoplasm and the cell wall and that this acts on the dye.

It would seem that this accelerating effect is due to a change in the nature of the dye (produced by the salt) which makes the dye penetrate more rapidly. It may not be necessary to suppose that the combination of salt plus dye affects the protoplasm so as to increase penetration because cells exposed[12] to such a combination behave subsequently like those exposed to salt without dye (showing a decrease in the rate of penetration when placed in the dye). We may therefore suggest that the effect of salt on the protoplasm is not affected by presence of dye. Further experiments are necessary, however, to determine this conclusively.

In striking contrast to the behavior of NaCl is that of salts with bivalent and trivalent cations. When cells are exposed to a solution of $MgCl_2$ and then placed in the dye solution there is no inhibiting effect such as is produced by previous exposure to NaCl. On the contrary, there is apparently an accelerating effect but this is so small that it may possibly be due to experimental error.

There is another difference[13] between these salts. The inhibiting effect of NaCl may be reversed by adding $MgCl_2$ to the solution containing NaCl or by washing the cells with a solution of $MgCl_2$ after they have been exposed to the solution of NaCl.

SUMMARY.

The effect of various substances on living cells may be advantageously studied by exposing them to such substances and observing their subsequent behavior in solutions of a basic dye, brilliant cresyl blue.

The rate of penetration of the basic dye, brilliant cresyl blue, is

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[12] From the time curves it is evident that the same amount of accelerating effect is produced whether the cells are placed in 0.02 M NaCl solution at pH 7.7 (borate buffer mixture) containing 14×10^{-5} M or 1.7×10^{-6} M dye. An inhibiting effect is produced when cells are exposed for 3 minutes to 0.02 M NaCl solution at pH 7.7 (borate buffer mixture) containing either 1.7×10^{-6} M dye or no dye, and then placed in 14×10^{-5} M dye solution at the same pH value for 1 minute.

[13] These differences may result from the fact that each salt affects the protoplasm or the dye in more than one way so that the net result may differ because with one salt one effect is of primary importance while with another salt a different effect predominates. One salt may affect diffusion from the external solution into the protoplasm a great deal while another may have more effect on the diffusion from the protoplasm into the vacuole and the location of these effects may change with alterations in concentration. The same may be said of differences in viscosity, solubility, etc.

decreased when cells are exposed to salts with monovalent cations before they are placed in the dye solution (made up with borate buffer mixture). This inhibiting effect is assumed to be due to the effect of the salts on the protoplasm.

This effect is not readily reversible when cells are transferred to distilled water, but it is removed by salts with bivalent or trivalent cations. In some cases it disappears in dye made up with phosphate buffer mixture, or with borate buffer mixture at the pH value in which the borax predominates, and in the case of NaCl it disappears in dye containing NaCl.

No inhibiting effect is seen when cells are exposed to NaCl solution containing $MgCl_2$ before they are placed in the dye solution.

The rate of penetration of dye is not decreased when cells are previously exposed to salts with bivalent and trivalent cations.

The rate is slightly increased when cells are placed in the dye solution containing a salt with monovalent cation and probably with bivalent or trivalent cations. In the case of the bivalent and trivalent salts the increase is so slight that it may be negligible.

THE STAGES OF THE PEPTIC HYDROLYSIS OF EGG ALBUMIN.

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Numerous investigators have studied the stages in peptic digestion. The conclusions at which they have arrived may be grouped under two main heads.

On the one hand, Kühne (1) and Neumeister (2) concluded that the products of digestion arise by a serial degradation from the protein through acid albumin to proteose to peptone. On the other hand, Lawrow (3), Klug (4), Pick (5), Zunz (6), and Goldschmidt (7) found that the products of digestion appear more or less simultaneously.

The most comprehensive study of the stages of peptic digestion was made by Zunz. His general conclusion was that metaprotein, proteoses (primary and secondary), peptones, and simpler products, arise simultaneously and by inference directly from the protein molecule. The metaprotein and a large part of the primary proteose, he found to be converted into peptones. He also described large amounts of substances (61 per cent of the total nitrogen) simpler than peptones which appeared in the early hours of digestion and later decreased.

Klug concluded that the acid metaprotein formation in the early stages of digestion was due to the action of pepsin, while Maly and Boos (8) believed that it might be due to the action of acid alone.

A reexamination of the problem seemed desirable. The methods used were chiefly those described by Wasteneys and Borsook (9) for the fractional analysis of incomplete protein hydrolysates.

Metaprotein (acid albumin) was estimated by determination of the nitrogen content of the precipitate obtained on careful adjustment of the reaction to pH 6.0.

The proteose was precipitated by saturation of the solution with sodium sulfate, at 33°C., and the peptone by precipitation with tannic acid under specially controlled conditions. The tannic acid filtrate was precipitated with alcohol and zinc sulfate. The nitrogen content of this precipitate was taken as subpeptone nitrogen, and the remainder as residual nitrogen, contained, it is assumed, in the simplest constituents of the hydrolysate.

No attempt was made to subdivide proteoses into primary and secondary classes except in one phase of the investigation which will be discussed later.

The results obtained are in accordance with those of Zunz and Pick that most of the products found in a peptic hydrolysate arise directly from the protein molecule. They differ in regard to the significance of acid metaprotein, and also in regard to the amount of, and variations in, the fraction simpler than peptone.

It was found that acid metaprotein is hydrolyzed more slowly than the native albumin from which it is derived; that the peptic hydrolysis of albumin can be effected at acidities less than pH 4.0, where no formation of metaprotein can be demonstrated; and that the hydrogen ion concentration for the minimum hydrolysis of undenatured albumin is distinctly different from that of acid metaprotein. For the former it is in the neighborhood of pH 6.0; for the latter at pH 4.0. From these results it was concluded that acid metaprotein formation, while a product of the action on protein of acid alone, as is well known, is not a necessary stage in the peptic hydrolysis of albumin, and its influence, if any, on hydrolysis, is to retard it.

Two-thirds of the initial amount of protein is hydrolyzed in 4 hours. The reaction then becomes progressively slower, so that in 12 hours 10 per cent, and in 7 days 6 per cent of the protein remains still unhydrolyzed. This rate of hydrolysis, however, holds only under the conditions of this experiment, but the rapid initial decomposition is characteristic of peptic hydrolysis under any conditions.

The results indicate that the peptic hydrolysis of albumin progresses in two stages. The first stage, occurring in the first 12 hours, consists of the hydrolysis of practically 100 per cent of the protein, with the formation of products of which 85 per cent may be spoken of as primary, *i.e.* undergo no further hydrolysis. The products at this time consist of 55 per cent proteose, 17 per cent peptone, 12 per cent subpeptone, and 5 per cent residual nitrogen. A second stage, occurring later and progressing much more slowly, results in the hydrolysis of 15 per cent of the primary products into simpler fragments, which may be designated as secondary. The secondary hydrolysis occurs in both the proteose and subproteose fractions.

No methods are available for following subsequent changes in the subproteose fraction. In any case they affect the main picture of peptic hydrolysis very little, as only a very small fraction, about 6 per cent at most, of the total N is involved.

EXPERIMENTAL.

Metaprotein Formation and Its Relation to Hydrolysis.

Egg albumin (Merck) and pepsin (Merck) have been employed throughout. The metaprotein was prepared by allowing a solution of albumin at pH 1 6 to stand at room temperature for several days, thymol being employed as antiseptic.

The rate of formation of metaprotein is shown in Table I. After several weeks it attains a value of about 80 per cent of the total N, leaving approximately 15 per cent of the nitrogen still in the form of albumin soluble at neutrality

A solution of metaprotein, on the other hand, prepared by solution at pH 1 6 of the precipitate obtained at pH 6 0 remains unchanged after standing for days at room temperature

To compare the relative rates of hydrolysis by pepsin of metaprotein, and of albumin which has not been denatured by acid, two simultaneous hydrolyses were followed. The first hydrolysis was of an albumin solution which had been standing at room temperature at pH 1 6 for several days. The second was of an albumin solution of equal nitrogen content, to which the enzyme was added simultaneously with an amount of acid necessary to bring the pH to 1 6. This experiment was performed with 2 0, 1 5, 1 0, 0 5, and 0 25 per cent stock pepsin solutions, lasting 45 minutes. Considering the lowest enzyme concentration as 1 unit the others were, respectively, 10, 8, 6, 4, and 2 units. The results are recorded in Fig. 1.

It is clear from Fig. 1 that in mixtures of undenatured albumin and acid metaprotein, the velocity of peptic hydrolysis is greater where the concentration of acid metaprotein is less. Fig. 1 also shows the surprising result, that, at least in these experiments, strict proportionality between velocity of hydrolysis and concentration of pepsin obtains in those solutions where the conversion of undenatured protein to metaprotein has attained approximate equilibrium

The slower rate of hydrolysis of acid metaprotein, whether the solution consists almost entirely of metaprotein, as in the previous experiment, or of a mixture of metaprotein and undenatured albumin, is shown in an experiment the result of which is indicated in Fig. 2. In this experiment the relative rates of hydrolysis were compared in three solutions of identical nitrogen and enzyme content, one containing undenatured albumin, a second consisting of equal parts of undenatured albumin and acid metaprotein, and a third containing only metaprotein. As the curves show, peptic hydrolysis is most rapid in the undenatured albumin solution, and least rapid in the metaprotein solution, while the relative rate of hydrolysis of the mixture is intermediate.

Since acid metaprotein was hydrolyzed more slowly than undenatured albumin, it seemed probable that the formation of metaprotein indeed was not, as Maly, and Boos (8) maintained, a necessary stage in peptic hydrolysis. To test this, experiments were performed to ascertain the possibility of obtaining peptic hydrolysis of albumin at hydrogen ion concentrations where no metaprotein is formed

TABLE I.

Rate of Formation of Acid Metaprotein from Egg Albumin at pH 1 6 and at 20°C.

Time	Metaprotein
	per cent total N
1 min.	10 6
5 "	17.0
20 "	18.9
30 "	21.3
1 hr.	26.5
2 "	33 3
6 "	39.0
1 day	54.6
3 "	56 9
5 "	68.6

It was necessary first to define the limit of acidity beyond which metaprotein formation does not occur Portions of a neutral solution of 3 2 per cent albumin were adjusted to various acidities with hydrochloric acid. These were set away in a water bath at 37.7°C for 45 minutes. They were then assayed for metaprotein by precipitation at pH 6 0. The results are given in Table II. They show that no metaprotein formation occurs at acidities less than pH 4.0.

As a result of this experiment a number of hydrolyses were carried out at acidities slightly less than pH 4 0 with several concentrations of pepsin. Typical results are given in Table III.

These experiments show that it is possible to effect considerable amounts of hydrolysis without the formation of any metaprotein. They also show that when hydrolysis is measured by following the changes occurring in the protein substrate, it is possible to detect the progress of hydrolysis at acidities less than pH 4.0. Michaelis (10) found that peptic hydrolysis of albumin ceases at pH 4.0.

The reason for the difference between our findings and those of Michaelis lies in the probability that the latter worker was dealing with a solution consisting largely of metaprotein. The hydrogen ion concentration for the threshold of minimum hydrolysis of acid metaprotein is at pH 4.0; for egg albumin, which has not been denatured, it is in the neighborhood of pH 6.0. The optimum pH is approximately the same for both, near pH 1.6. This is shown in Fig. 3. In this experiment 3.2 per cent solutions of albumin and of acid

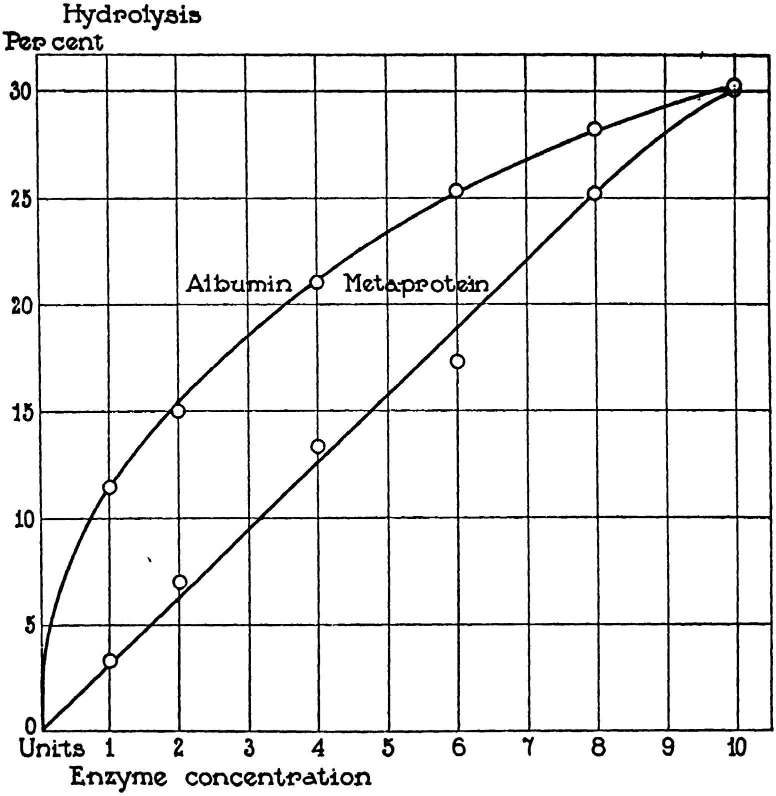

FIG. 1. Relative rates of hydrolysis of albumin and metaprotein.

metaprotein were hydrolyzed for 1 hour at 37.7°C. with 0.2 per cent pepsin at the acidities indicated.

In the peptic hydrolysis of coagulated egg albumin observations were made, which, in contradistinction to the findings with uncoagulated albumin, do not conform to the view that acid metaprotein is not a necessary stage in the peptic hydrolysis of albumin. A 3.2 per cent solution of albumin, acidified with dilute acetic acid, was coagulated by boiling. The coagulate was filtered and washed thoroughly with distilled water. It was then suspended in dilute HCl, and the reaction of the suspension adjusted to pH 1.6. Pepsin was added to a concentration of 0.2 per cent, and the suspension was incubated at 37.7°C. for 3 hours. At the end of this time all of the coagulum had disappeared. Part of the digest was neutralized;

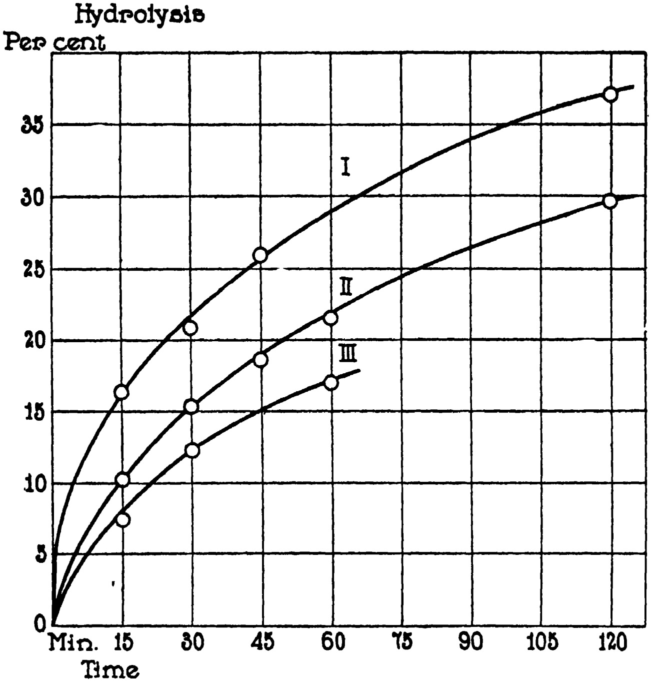

FIG. 2. Relative rates of hydrolysis of albumin and metaprotein. I, albumin; II, mixture of albumin and metaprotein; III, metaprotein.

another part was treated with trichloroacetic acid. In the neutralized solution a heavy precipitate appeared. The total nitrogen of the solution was 42 mg. The nitrogen in the filtrate from trichloroacetic acid precipitation was 30.6 mg. indicating that 11.4 mg. of protein nitrogen had not been hydrolyzed. The filtrate of the neutralized solution contained 31.9 mg. of nitrogen. The precipitate thrown out on neutralization contained, therefore, 10.1 mg. The nitrogen precipitated by trichloroacetic acid was 11.4 mg., by neutralization 10.1 mg. In view of the difference of the two methods the correspondence is sufficiently close to indicate that the same amount of material was precipitated by neutralization and by tri-

TABLE II.

Relation of Acid Metaprotein Formation to the C_{H^+}.

pH	Amount of metaprotein formed
	per cent total N
6.0	0
4.5	0
4.3	0
4.2	0
4.1	0
4.0	0
3.9	3.9
3.5	21.6

TABLE III.

Peptic Hydrolysis of Egg Albumin at Acidities Less than pH 4.0.

Enzyme concentration	pH 4.1		pH 4.2	
	Amount of hydrolysis	Metaprotein formed	Amount of hydrolysis	Metaprotein formed
units	*per cent*		*per cent*	
1	5.3	0	2.8	0
2	6.3	0	—	—
4	6.5	0	—	—
6	8.2	0	3.4	0
8	10.6	0	—	—
10	10.7	0	4.4	0

chloroacetic acid. Evidently there is a material produced in the early stages of the hydrolysis of coagulated egg albumin which is soluble in dilute acid, insoluble at the neutral point, and precipitated by trichloroacetic acid. This agrees with the definition of metaprotein. We are led to the conclusion, for the time being, that though metaprotein is not a stage in the peptic hydrolysis of soluble undenatured albumin, it is probably the first stage in the hydrolysis of coagulated egg albumin.

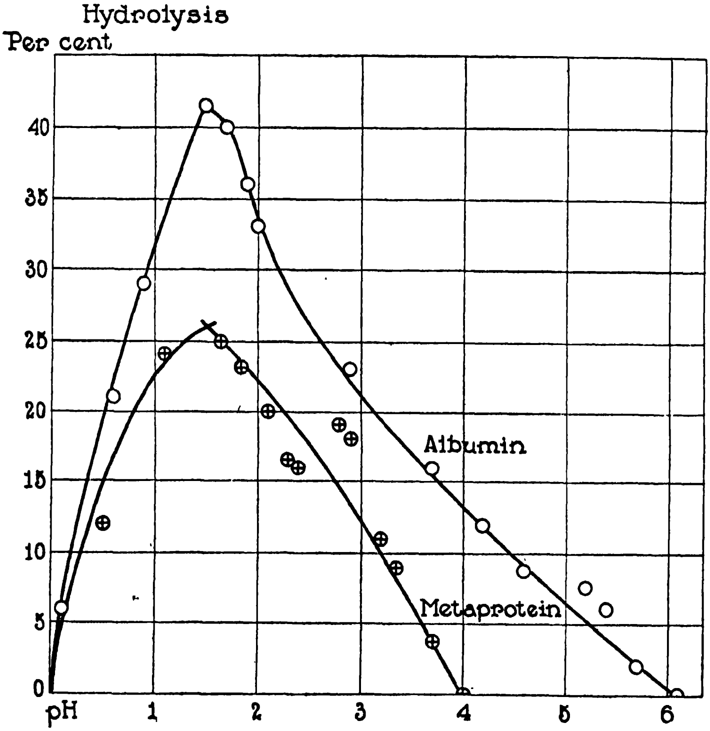

FIG. 3. Relative effect of C_{H^+} on the rates of hydrolysis of albumin and metaprotein.

The phenomena associated with heat coagulation of albumin have been fully discussed by Wu and Wu (11). This experiment, however, discloses an interesting anomaly. It is ordinarily supposed that in the coagulation of egg white by heat, albumin passes through the stage of acid metaprotein. On suspending the coagulum in acid so that the reaction of the suspension is pH 1.6, contrary to the behaviour of uncoagulated albumin, no metaprotein is formed. Immediately on adding pepsin, however, metaprotein appears. Either the change from metaprotein to coagulated egg white is a reversible phenomenon with pepsin as a necessary adjuvant for reversion, or else we are dealing with different kinds of metaprotein.

The Stages in the Peptic Hydrolysis of Albumin.

The method of analysis was that described by Wasteneys and Borsook (9) for the fractional analysis of incomplete protein hydrolysates. Two types of experiments were carried out. One was a preliminary survey, in which the pH of the digest was allowed to rise with the progress of the hydrolysis. In the other, the pH was kept approximately constant at 1.6 throughout the course of the experiment by the occasional addition of acid. The findings in both types of hydrolyses were essentially the same, but the latter experiment was more thoroughly controlled, and the results there obtained are taken as the basis for the present discussion.

6 litres of a 5 per cent solution of egg albumin (Merck) were adjusted to pH 1.6 and sufficient solution of scale pepsin (Merck), also adjusted to pH 1.6, was added to make the concentration of enzyme 0.2 per cent.

The following control experiments were carried out. 1000 cc. of the albumin solution at pH 1.6 was precipitated with trichloroacetic acid and analyzed at the same time as the first sample from the digest. A simultaneous analysis was made of another 1000 cc. of the same albumin solution at pH 1.6 which had been in the incubator with the main digest for a week. These two controls determined the effect of the acid alone (pH 1.6) on egg albumin during the period of the experiment (168 hours). The protein N diminished from 96 per cent of the total nitrogen to 94 per cent. The proteose and residual nitrogen fractions remained unchanged. The peptone rose from 2 to 3 per cent and the subpeptone from 0 to 0.5 per cent. The proteolytic effect of the acid is therefore negligible. This is in accord with the conclusions of Frankel (12) who found no significant increase in the free amino nitrogen of egg white after incubation for 96 hours with 0.2 per cent HCl at 38–40°C.

In the presence of pepsin, as Table IV shows, the hydrolysis of the protein was very rapid. In 4 hours more than two-thirds of the protein was hydrolyzed; in 12 hours 11 per cent of the original total amount of substrate remained. After this point the hydrolysis of the remaining protein proceeded slowly. For the complete hydrolysis of 3 per cent albumin at 37°C. with this enzyme concentration, between 2 and 3 weeks are required.

The proteose fraction attained a maximum value of 55 per cent. Between 12 and 28 hours, the amount of this fraction falls from 55 per cent to 50 per cent; and in addition there is a further hydrolysis of 6 per cent protein. During this interval the peptone fraction gains 12 per cent, from 17 per cent to 29 per cent, while the subpeptone value increases only 3 per cent. The increase in residual nitrogen is negligible. The relatively greater gain in the peptone fraction is better shown by the curves, Fig. 4. At 12 hours, when the proteose curve has attained its peak, the slope of the subpeptone curve changes from a relatively steep ascent to a very slowly rising straight line. The peptone curve, on the other hand, continues to rise fairly quickly until 28 hours, when it flattens out. After 28 hours the proteose decreases from 50 per cent to 40 per cent; the peptone curve is practically flat, while the subpeptone rises from 15 to 23 per cent.

TABLE IV.

Fractional Analyses of a Peptic Digest of Albumin during the Course of Almost Complete Hydrolysis.

Duration of hydrolysis	Protein N	Proteose N	Peptone N	Subpeptone N	Residual N
	Per cent of original protein N				
3 min.	85	6	6	2	1
12 hrs.	11	55	17	12	5
28 "	5	50	29	15	2
48 "	5	46	29	18	5
72 "	4	45	30	19	5
96 "	5	45	31	17	5
168 "	6	40	27	23	7

The peptone fraction rises rapidly to a value of 24 per cent in 28 hours. The increase between 12 and 28 hours is probably due, as discussed above, to the hydrolysis of proteose during this interval. After 28 hours the value of the peptone remains practically constant at approximately 24 per cent. This value probably represents an equilibrium between the amount gained from the hydrolysis of proteose and that lost by secondary hydrolysis of the peptone itself.

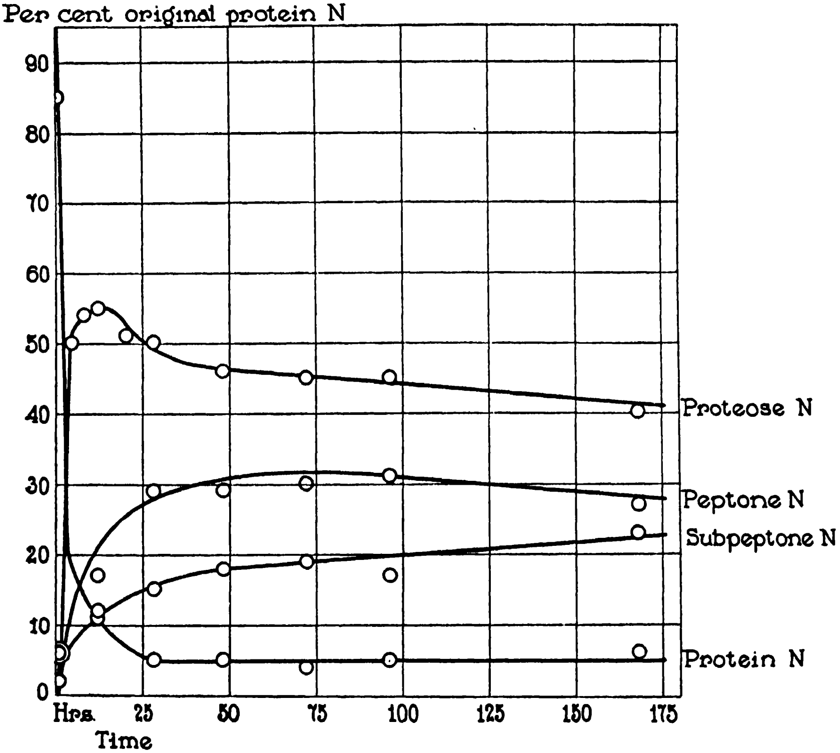

FIG. 4. Fractional composition of the hydrolysate during the course of almost complete peptic hydrolysis of egg albumin.

The subpeptone rises to a value of 10 per cent in 12 hours. The subsequent increase is slow and becomes significant only in the later periods of the hydrolysis, rising to 19 per cent in 168 hours. There is probably very little, if any, subpeptone hydrolyzed by pepsin. Hence this fraction continues to increase steadily, gaining, by secondary hydrolysis, from the proteose and peptone.

The increase in residual nitrogen, though small, from 1 to 4 per cent, was obtained in a number of digests, and is larger than can be accounted for as experimental error. As Table IV shows, an increase in residual nitrogen is obtained very early in the hydrolysis.

These results suggest that the secondary hydrolysis is of a serial type, similar to that which the older writers considered to be the nature of the whole hydrolytic process.

In order to study the secondary hydrolysis more closely, a 3.2 per cent solution of egg albumin was digested with pepsin, and samples were removed after 3 minutes and 6, 12, 24, 48, and 72 hours. The protein and proteose were determined at these intervals, and at the same time 500 cc. samples were removed, and the proteose and subproteose fractions isolated from them. The method for the separation of these factions consisted in precipitation of protein with trichloroacetic acid, boiling of the filtrates to decompose trichloroacetic acid, neutralization, and dilution to volume. The solutions were then saturated with sodium sulfate at 33°C. The precipitate was taken as the proteose fraction and the filtrate as the subproteose fraction. The salt was removed from the latter fraction by adding alcohol to 50 per cent. The filtrate from the precipitated salt was evaporated down to drive off alcohol, and the residue was made up to volume.

Both proteose and subproteose fractions were then adjusted to pH 1.6 with 2.0 N HCl. Pepsin to a concentration of 0.2 per cent was added and the increase in formol titrable N was followed at intervals of 3 days and 7 days.

It is generally agreed in the literature that copper acetate is a specific precipitant for primary proteose. Both proteose and subproteose fractions were therefore treated with copper acetate according to the method of Folin (13), at intervals of 3 days and 7 days, simultaneously with the formol titrations.

Considering first the fraction precipitated by copper acetate, it was found to be present in both proteose and subproteose, which makes the specific nature of the copper acetate precipitation doubtful. However, the amount of N so precipitated in the proteose fraction removed from the earlier stages of the primary hydrolyses was greater than in the fractions removed later, and to an extent that might account for the total falling off in proteose from the maximum attained during the primary hydrolysis. It is significant in this regard that there is no change in the N precipitated by copper acetate in any of the subproteose fractions.

From this it may be inferred that though copper acetate is not a specific precipitant of primary proteose, yet it appears to precipitate all of the proteose which undergoes secondary hydrolysis.

In the second digestion by pepsin the changes in free amino N, as shown by the formol titration, were relatively greatest in the earliest obtained proteose fractions. The later samples of proteose and all the subproteose fractions gave relative increases of approximately equal magnitude.

The conclusions are that secondary hydrolysis takes place in both fractions. It occurs to a greater extent in the earlier proteose fractions coinciding with the fall in the proteose curve. In relation to the main hydrolysis, however, these increases in free amino N, in both fractions, are small, and indicate the minor importance of the secondary hydrolysis in the peptic digestion of albumin.

SUMMARY.

1. Most of the products of the peptic hydrolysis of albumin, about 85 per cent of the total N, are primary in the sense that they arise directly from the protein molecule, and undergo no further hydrolysis.
2. A slow secondary hydrolysis, involving about 15 per cent of the total N, occurs in the proteose and simpler fractions primarily split off.
3. Acid metaprotein in peptic hydrolysis arises as a result of the action of acid. It is not an essential stage in the hydrolysis of undenatured albumin.
4. Acid metaprotein is hydrolyzed by pepsin more slowly under comparable conditions than undenatured albumin.

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THE AUTODESTRUCTION OF PEPSIN IN RELATION TO ITS IONIZATION.

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The irreversible disappearance of the activity of pepsin in alkaline solution was first recorded by Kühne (1). It was confirmed by Langley (2), who observed also "that the destruction by sodium carbonate solutions goes on very much more slowly at 15°C. than at body temperature." Langley's experiments were carried out with neutralized filtrates of extracts of rabbit gastric mucosa made with 0.1 per cent HCl. He found no especial differences in the pepsins from the cat, dog, mole, newt, and snake. He mentions a possibility of frog pepsin being destroyed more rapidly than that of mammals. Biernacki (3) observed that the proteolytic activity of gastric juice was retained at a higher temperature in acid solution than at neutrality. This author found also that the resistance to destruction by heat was increased by the presence of peptones, and that a pure preparation was accordingly less stable than the pepsin in gastric juice. Grober (4) concluded from the average of a number of determinations that urinary pepsin heated with 0.25 per cent HCl was destroyed at 66°C., while pepsin from the same source, dissolved in water, was destroyed at 64°C. This difference he interpreted as indicating a chemical combination between the acid and the pepsin. The combination was thought to retard the hydrolysis of the pepsin. This mechanism was considered by Grober to be the cause of the destruction of pepsin by heat.

Michaelis and Rothstein (5) investigated the kinetics of the destruction of rennin and pepsin. They were unable to distinguish between these two properties of gastric juice preparations by their behaviour towards alkali. They derived an empirical differential equation in which the velocity of autodestruction was proportional to the fourth power of the hydroxyl ion concentration. They found that at 25°C. and at 35°C., at reactions more acid than pH 6.0, the enzyme suffers no loss in potency, and that on approaching neutrality from the acid side the velocity of destruction suddenly becomes increasingly more rapid, until, in faintly alkaline solutions, it is too great to be measured. Michaelis and Rothstein found that the autodestruction at any given hydrogen ion concentration was not monomolecular. The velocity at constant hydrogen ion concentration in their experiments was proportional to the one and one-half power of the enzyme concentration. The destroyed pepsin was without influence upon the destruction of the remaining active enzyme, from which Michaelis and Rothstein concluded that the reaction is irreversible.

Northrop (6) found that pepsin solutions were most stable at 38°C. at a hydrogen ion concentration of 10^{-5}. "Neither the purity of the enzyme nor the anion of the acid used exerted any marked influence upon the rate of destruction, or on the zone of hydrogen ion concentration in which the enzyme was most stable."

The ionic nature of pepsin was suggested by Loeb in 1909 (7).

These studies of the autodestruction of pepsin are unanimous in their agreement upon the rapid destruction of the enzyme in acidities less than pH 6.0 The purpose of the investigation described below was to discover, if possible, the mechanism of the action of the OH ion. The method differed from that of other workers in that the amount of active enzyme present, at any given hydrogen ion concentration, was determined for zero time. This procedure is facilitated by the fact that the logarithms of the amounts of active enzyme present give a straight line when plotted against the time that the pepsin has been subjected to the hydroxyl ion concentration under consideration.

The zero values at 15°C. so obtained, plotted against pH, fall about a curve which represents the dissociation of a univalent compound with a value for pK of 6.85. The conclusion seems permissible that pepsin is such a univalent compound. This is in accordance with the conclusion of Northrop (8), attained by a totally different method, that pepsin is a univalent compound in the range of hydrogen ion concentrations from pH 1 to pH 7.

When the initial amount of enzyme is taken to be the extrapolated value found for active enzyme at zero time, results at 20°C. and 37°C., with those obtained at 15°C., showed that the autodestruction of pepsin is a monomolecular reaction. Michaelis and Rothstein assumed that at all hydrogen ion concentrations 100 per cent of the enzyme was active at zero time. This assumption, however, in the light of the experimental data given below seems to be unsound.

The products of the autodestruction of pepsin exert no inhibiting influence on the rate of subsequent autodestruction. This conclusion is drawn from the absence of any constant diminishing tendency in the values for K, and is similar to that of Michaelis and Rothstein.

The velocity of autodestruction is directly proportional to the hydroxyl ion concentration, when this is greater than pOH 7.7. This linear relationship is shown in Table V and Fig. 3.

It is difficult to interpret the variations in K over the whole range of hydroxyl ion concentrations at which the autodestruction was followed. The curve obtained may represent two different processes, or one process somewhat similar to the neutralization of a strong acid with a strong base.

The relation of autodestruction to ionization and the monomolecular decomposition suggest the following outline of the mechanism involved in the autodestruction of pepsin.

There are two independent processes, both resulting in irreversible loss of enzyme activity. The first is the ionization of the pepsin, with the two obvious possibilities of pepsin as either a weak acid or a weak base. If it is a weak acid, Fig. 2 represents the undissociated residue of the enzyme; and it follows that it is the undissociated form of the enzyme which is active in hydrolysis. If pepsin is a weak base, on the other hand, then the curve represents the degree of dissociation, and it is the pepsin ion which is active. For the purposes of discussion the enzyme will be considered as a weak acid and the curve will be taken to represent its undissociated and hydrolytically active residue. It is the ionized form, therefore, which must be considered to undergo the irreversible change which results in the destruction of the enzyme. This occurs at zero time, *i.e.* as soon as the alkali is mixed with the pepsin. The progressive falling off from 100 per cent, with increasing hydroxyl ion concentration, of the extrapolated values at zero time, makes it necessary to consider this change irreversible. Since ionization is completely reversible, another subsidiary reaction must be considered to occur, possibly some stereoisomeric transformation, from which the original, undissociated hydrolytically active form, cannot be regained on restoration of the acidity to pH 1.6.

This transformation must permit the newly formed, irreversible compound to function in the ionic equilibrium as well as its precursor. Otherwise the second component of the process of autodestruction about to be discussed could not be defined by the equation for a monomolecular reaction.

The first process can be represented then by the following equations.

$$(1)\quad \mathrm{H\,A} \rightleftarrows \mathrm{H}^{+} + \mathrm{A}^{-}$$

$$(2)\quad \mathrm{A}^{-} \rightarrow \mathrm{A}_{1}$$

Equation (1) represents ionization and (2) the instantaneous, irreversible, complete conversion of the anion into an isomer, which, while it may participate in the ionic equilibrium, does not, on recombination with H^+, form active enzyme.

The second mode of irreversible loss of activity of the pepsin can be represented by the equation,

$$(3)\quad \mathrm{H\,A} \rightarrow \mathrm{B}$$

As discussed above, this reaction is defined by the mass law equation for a monomolecular reaction.

The original amount of active enzyme; *i.e.*, a, in the equation $K = \frac{1}{t} \log \frac{a}{a - x}$, is the extrapolated value of active enzyme at zero time.

This quantity is represented by the amount of H A at any given pH at zero time as shown in equation (1). If reaction (3) were dependent upon (1) and (2), then as H A was converted to B, equation (1) would be reversed in order to form H A from H^+ and A^-. If this happened then (3) could not be defined by the monomolecular equation.

The mechanism of this independence of (3) from (1) is probably represented by equation (2) which does not permit the reversion of any of A to H A. This possibility suggested; *viz.*, the formation of an isomer, which, while incapable of taking part in the reconstitution of the hydrolytically active form of the enzyme, may nevertheless function in ionic equilibrium, does not appear capable of direct experimental verification. A similar conception was advanced by Northrop (9).

The scheme involves two consequences. The first is, that no matter how low the temperature at which a solution of pepsin be maintained, if the C_{H^+} be lowered below that required for minimum ionization, there is an immediate and irreversible destruction of more or less of the pepsin depending on the pH. The second consequence is that the relation of the progressive autodestruction to the C_{OH^-} must be independent of the degree of dissociation of the pepsin, *i.e.* must show no relation to the dissociation curve. Both of the consequences are found in practice.

The first is demonstrated in the fact that an extrapolated value of 100 per cent is not obtained at any pH at which dissociation occurs. The second is indicated by the lack of any obvious relationship between Figs. 2 and 3.

EXPERIMENTAL.

In the experiments at 15°C., egg albumin (Merck) and scale pepsin (Wyeth) were employed. In those at 20°C., and at 37°C., which were performed 2 years previously, egg albumin (Merck) and scale pepsin (Merck) were used.

A 10 per cent solution of pepsin was made in N/10 HCl, giving an acidity approximating pH 3. An electrometric titration was carried out with N/1 NaOH to ascertain the amount of alkali required to bring the pepsin solution to a desired pH. For the autodestruction experiments 10 cc. of the stock 10 per cent pepsin solution was measured into a 50 cc. volumetric flask in a water bath at 15°C. Distilled water employed for dilution, and the N/10 NaOH, were also brought to 15°C. The calculated amount of alkali was added to the volumetric flask followed by distilled water up to the mark. The flask was shaken, and the contents transferred to a 100 cc. Erlenmeyer flask which was stoppered and placed in the water bath at 15°C. At the recorded times 5 cc. of this solution was removed with a pipette and added to 50 cc. of albumin solution at pH 1.6 and at 30°C. The hydrolysis was allowed to proceed for 1 hour when 50 cc. of the digest was removed and pipetted into 12.5 cc. of 10 per cent trichloroacetic acid. The total nitrogen of the filtrate, after deducting the appropriate blanks, was taken as representing the amount of protein hydrolyzed. After allowing for dilution, the percentage of active enzyme was calculated from the amount of hydrolysis effected by 5 cc. of similar pepsin solution which had been diluted with water only, and to which no alkali had been added. Autodestruction was assumed to have commenced from the moment at which the NaOH was added to the pepsin.

The hydrogen ion concentration was measured both electrometrically and colorimetrically. M/10 phosphate solutions with methyl red, brom-cresol purple, and brom-thymol blue as indicators, were employed as colour standards. The colour of the pepsin solution was not deep enough to interfere with colorimetric estimations. Throughout the range from pH 5.5 to 7.15 the electrometric and colorimetric readings were identical.

On account of the relatively high concentration of albumin employed, 3.2 per cent, it was not necessary to add a buffer to the protein solutions undergoing hydrolysis. With the most active pepsin solution the pH, after 1 hour's hydrolysis at 30°C., rose only from 1.6 to 1.7.

Three controls were used for each hydrogen ion concentration; the estimation of total non-protein nitrogen of albumin and of pepsin, and the determination of the amount of hydrolysis effected by enzyme which had been diluted only with water, *i.e.* had undergone no autodestruction. In the results given in Table I and Fig. 1 these controls have been deducted. The amounts of active enzyme were calculated from the proportion between the amounts of hydrolysis obtained with the enzyme partially destroyed and that effected by enzyme of unimpaired activity.

At each C_{H^+} the extrapolated zero value ((a) in Table I) is therefore stated as a proportion of the original unimpaired activity of the enzyme. Similarly, the values given under $(a - x)$ (Table I) represent the amounts of hydrolysis obtained with partially destroyed enzyme. K (Table I) is calculated by means of the mass law equation for a monomolecular reaction. In Columns (a) and $(a - x)$, 1 per cent represents about 0.15 to 0.3 mg. of N in the macro-Kjeldahl determination.

In Fig. 1 are plotted the logarithms of the amounts of active enzyme

TABLE I.

The Effect of C_{H^+} on Autodestruction of Pepsin at 15°C.

pH	Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a-x}$	Average value for K
	min.	*per cent of original amount*	*per cent of original amount*		
4.35	10	99	99.6	.0002	.0004
	20	96.3		.0007	
	40	96.0		.0004	
	50	94.7		.0004	
4.6	10	(96)	99	—	—
	20	100		—	
	30	98		—	
	40	98		—	
	50	100		—	
5.2	10	95	96	—	—
	20	(91)		—	
	30	96		—	
	50	(99)		—	
	60	96		—	
5.9	10	88.5	88.8	.0002	.0002
	20	(90)		—	
	30	86		(.0007)†	
	40	87.5		.0002	
	50	87.6		.0001	
5.9	10	82	80.9	—	.0005
	20	79		.0005	
	30	78		.0006	
	40	77.6		.0005	
6.22	10	80	80.7	(.0004)	.001
	20	77.5		.001	
	30	75.4		.001	
	40	73.6		.001	
	50	72.0		.001	
6.4	10	71	73.3	.0014	.0014
	20	69		.0013	
	30	66		.0015	

TABLE I—*Concluded.*

pH	Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average value for K
	min.	*per cent of original amount*	*per cent of original amount*	†	
6.54	10	68.3	67.9	—	.0007
	20	66.0		.0007	
	40	63.1		.0008	
	50	62.8		.0007	
6.64	15	57.7	58.2	(.0003)	.0014
	30	52.2		.0015	
	45	50.6		.0014	
	60	48.8		.0013	
6.75	10	46	56.2	.009	.011
	20	33.5		.011	
	30	23		.013	
	40	19		.012	
	50	16		.011	
6.85	10	52.2	69.3	.012	.012
	20	37.4		.013	
	30	28.7		.013	
	40	21.3		.013	
	50	18.8		.011	
6.97	4	29.6	44.2	.044	.045
	8	17.9		.049	
	12	12.9		.045	
	16	9.3		.042	
	20	5.3		.046	
7.15	2	18.3	26.3	(.08)	.17
	4	5.5		.17	
	6	2.5		.17	
	8	0.9		.18	
	10	0.6		.16	
7.35	1.25	4	23.2	.61	.66
	2	1		.68	
	3	0.2		.69	

* Obtained by extrapolation (Fig. 1).

† Bracketted values for K were not used in computing the average value for K.

present against the length of time that the pepsin solutions were subjected to the respective hydroxyl ion concentrations. These plots give straight lines, as mentioned above, and are produced to zero time.

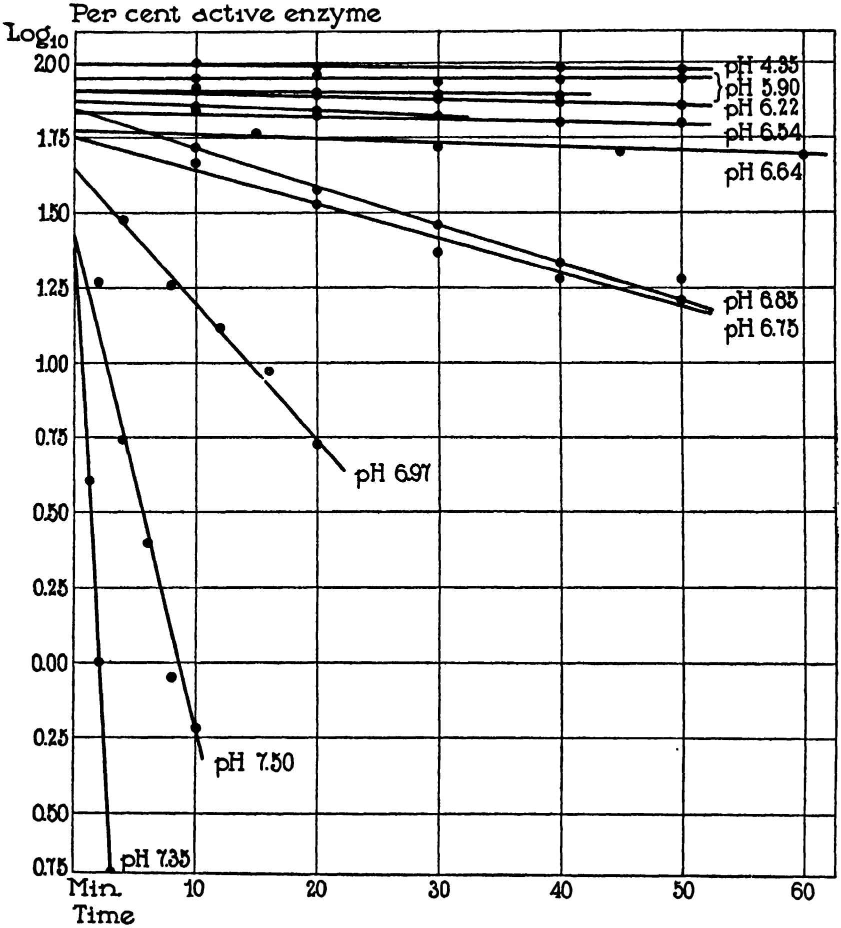

FIG. 1. The autodestruction of pepsin at 15°C. with time, at various reactions. The points are the logs_{10} of amounts of active enzyme (in per cent of original amount). The values for the per cent of active enzyme at zero time are plotted against pH in Fig. 2.

In Tables II and III are given results of autodestruction experiments carried out at 20°C. and at 37°C. The pH values are uncertain and hence these cannot be compared with experiments at 15°C. They

TABLE II.

Autodestruction of Pepsin at Different Hydrogen Ion Concentrations More Alkaline than pH 6.0 and at 20°C.

Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average K
min.	*per cent of original amount*	*per cent of original amount*		
3	75.9	81.3	.010	.011
6	69.2		.011	
9	64.6		.011	
12	60.3		.011	
3	36.3	44.7	(.030)†	.041
6	25.1		.042	
9	19.1		.041	
12	15.1		.039	
15	10.5		.042	

* Obtained by extrapolation.

† Bracketted values for K were not used in computing the average value for K.

TABLE III.

Autodestruction of Pepsin at Different Hydrogen Ion Concentrations More Alkaline Than pH 6.0 and at 37°C.

Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (x)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average K
min.	*per cent of original amount*	*per cent of original amount*		
15	33	62.4	.018	.017
30	19.6		.017	
105	1.0		.017	
3	36.8	50.1	.045	.045
9	19.2		.046	
12	15.2		.043	
15	10.4		.046	
3	29.6	39.8	.043	.056
6	18.4		.056	
9	12.0		.058	
12	8.8		.055	
15	4.0		.067	

* Obtained by extrapolation.

show, however, that at a given pH the course of the autodestruction of pepsin at these temperatures is defined by the monomolecular equation as at 15°C.

In Table IV are given, for various hydrogen ion concentrations, the values for the undissociated residue of an acid with pK 6.85. These are compared with the extrapolated values of active enzyme at zero time at the same reaction. The data in Table IV are depicted graphically in Fig. 2.

TABLE IV.

Amounts of Active Enzyme at Zero Time Compared with the Dissociation of an Acid, pK 6.85.

pH	Active enzyme at zero time	Undissociated residue of acid
	per cent	*per cent*
4.35	99.6	99.7
4.6	99.0	99.0
5.2	96.0	97.8
5.9	80.9	89.9
5.9	88.8	89.9
6.22	80.7	81.0
6.4	73.3	73.8
6.54	67.9	67.1
6.64	58.2	61.9
6.75	56.2	55.7
6.85	69.3	50.0
6.97	44.2	43.2
7.15	26.3	33.4
7.35	23.2	24.0

In spite of the experimental difficulty of obtaining close agreement in the amounts of active enzyme at zero time, as evidenced by the disagreement in the results for two different experiments at pH 5.9 (Table I) and other discrepancies, the majority of the values obtained fall closely about the ideal dissociation curve (Fig. 2).

These discrepancies do not, in any case, affect the constancy of the value for K at any pH, though they may alter its absolute value, and are insignificant when the whole range of variation of K with pOH is considered.

In Table V are given the average values of K in Table I, the values for pK, and the corresponding hydroxyl ion concentrations. By extrapolation from the data given by Michaelis (10) the value for pK_w at 15°C. was taken as 14.24. This value minus the pH gives the pOH.

In Fig. 3 are plotted the values of pK against pOH. The curve shows a direct proportionality between the velocity of autodestruction and C_{OH^-} in the range of concentrations greater than pOH 7.7; and possibly another proportionality, also direct, in the range from beyond pOH 9.89 to 7.7.

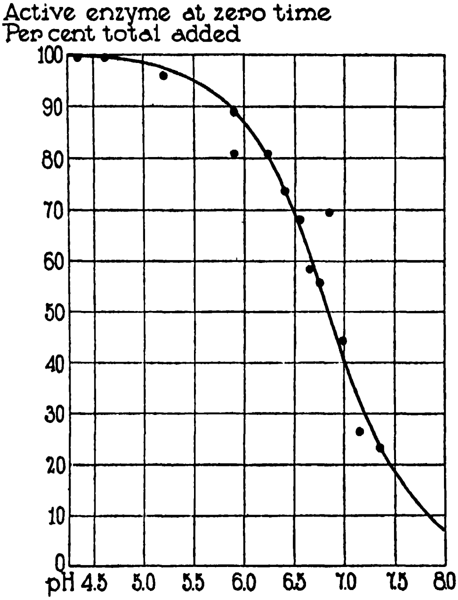

FIG. 2. The ideal dissociation curve of an acid with pK 6.85. The points are obtained from experimental data. They are amounts of active enzyme at zero time for the noted pH values and are obtained by extrapolation (Fig. 1).

Not all the experiments recorded in Table I were carried out with the same albumin solution. In those at pH 4.35, 5.9, 6.22, and 7.35 undenatured albumin was employed, while acid metaprotein served as substrate for the action of the pepsin in the experiments at pH 4.6, 5.2, one at 5.9, 6.4, 6.54, 6.64, 6.75, 6.85, 6.97, and 7.15. In the earlier experiments at pH 6.54, 6.64, 6.85, 6.97, and 7.15 the existence of a strict linear proportionality between the amount of hydrolysis in 1 hour at 30°C. and the concentration of pepsin was assumed. This assumption was made on the basis of results obtained by McFarlane, Dunbar, Borsook, and Wasteneys (11) with acid metaprotein as substrate. They found denatured albumin to give linear proportionality between velocity of hydrolysis and concentration of enzyme. It was found later that this result could not invariably be obtained. This was the case with the denatured albumin employed in the present experiments. An error is therefore introduced in the computations of results in the five experiments mentioned. It is believed, however, that it does not invalidate the conclusions, because under the experimental conditions the error introduced is not great.

TABLE V.

Relation of the Velocity of Autodestruction, K, to the Hydroxyl Ion Concentration.

pH	pOH	*K*	pK
4.35	9.89	.0004	3.40
4.6	9.64	—	—
5.2	9.04	—	—
5.9	8.34	.0005	3.30
5.9	8.34	.0002	3.70
6.22	8.02	.0010	3.0
6.4	7.84	.0014	2.85
6.54	7.70	.0007	3.15
6.64	7.60	.0014	2.85
6.75	7.50	.011	1.96
6.85	7.39	.012	1.92
6.97	7.27	.045	1.35
7.15	7.09	.17	0.77
7.35	6.89	.66	0.18

Empirical reference curves were employed in calculating the amount of active enzyme in the majority of the experiments. The data for the reference curve for experiments at pH 4.35, 5.9, 6.22, and 7.35 are given in Table VI and the curve is shown in Fig. 4.

The procedure was as follows: varying amounts of 2 per cent pepsin were added to 50 cc. of 3.2 per cent undenatured albumin at pH 1.6. In order to maintain the nitrogen content the same with each concentration of enzyme the pepsin was diluted with an inactivated solution of the same concentration. The stock pepsin solution was brought to pH 7.5 at room temperature for 10 minutes, then acidified to pH 1.6 and diluted to a final concentration of 2 per cent. The amount of hydrolysis at 30°C. for 1 hour was measured in the usual manner by precipitation of the unhydrolyzed protein with trichloroacetic acid, and estimation of the nitrogen content of the filtrate.

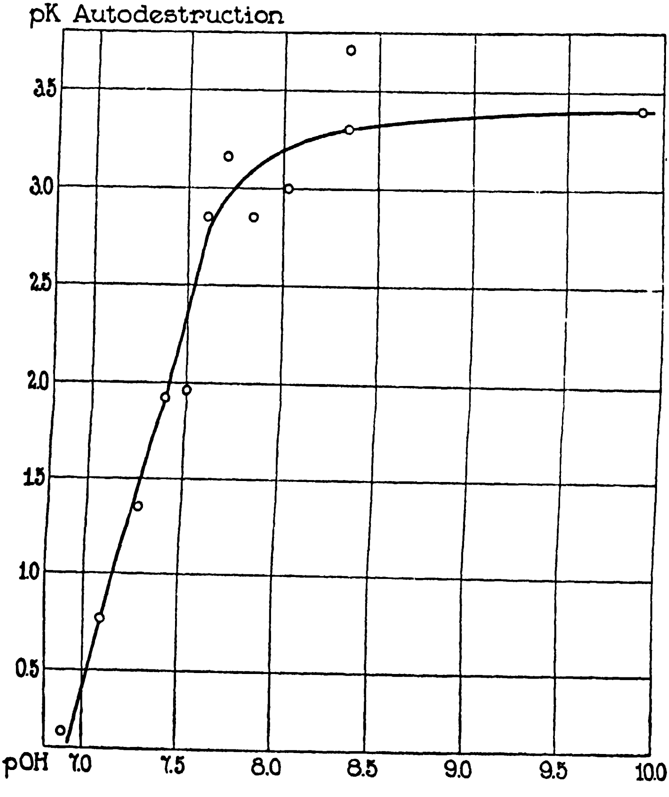

FIG. 3. The relation of the velocity of autodestruction to C_{OH^-} plotted as pK against pOH.

For experiments at pH 4.6, 5.2, one at 5.9, 6.4, and 6.75, acid metaprotein was used, and another reference curve.

The results obtained with the three solutions of albumin are incorporated into one body of data. The values for active enzyme at zero time, whether obtained with acid metaprotein or with undenatured albumin fall near one dissociation curve as shown in Fig. 2. When

TABLE VI.

Relation between Amount of Hydrolysis in 1 Hour at 30°C. and the Concentration of Pepsin.

2 per cent active pepsin	2 per cent inactive pepsin	3 2 per cent albumin	Albumin N hydrolyzed
cc	*cc.*	*cc*	*mg.*
5	0	50	77.6
4	1	50	69 8
3	2	50	59 2
2	3	50	46.8
1	4	50	32.6

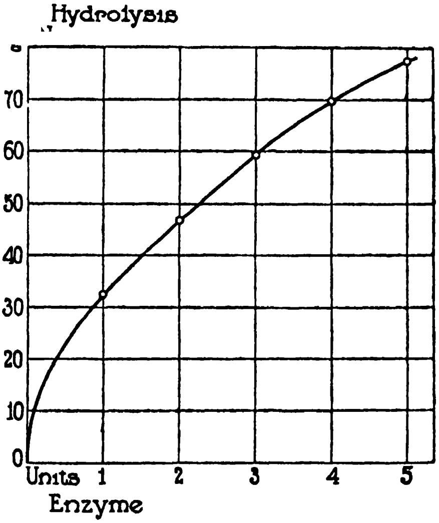

FIG. 4. A reference curve. The relation between the amount of hydrolysis (mg. of N) in 1 hour at 30°C. and the concentration of pepsin (cc. of 10 per cent solution).

the various quantities of active enzyme, calculated in each case according to the nature of the substrate, are inserted into the monomolecular equation, a practically constant value for K is obtained at each hydrogen ion concentration. These values for K as Fig. 3 shows, vary, in general, with the hydroxyl ion concentration, regardless of the kind of albumin used in the particular experiment. These coincidences give added support for the validity of the methods employed for calculating active enzyme.

Observations made in the course of these experiments demonstrate the need for caution in comparing results obtained with different solutions of presumably the same albumin. The undenatured albumin in the experiments described here was hydrolyzed by a given solution of pepsin nearly three times as quickly as a similar solution of albumin which had been standing at pH 1.6 at room temperature for several weeks. Any relationship found between velocity of hydrolysis and enzyme concentration with a given solution of albumin may not be assumed to obtain with any other similar solution subjected to even slight changes in previous treatment. In order to obtain directly comparable results, the previous history of the albumin used for every determination must be identical.

SUMMARY.

1. Evidence is presented that pepsin is a univalent acid with a value for pK of 6.85 (or a base, with pK 7.39).

2. The autodestruction of the pepsin is shown to be dependent in part upon an instantaneous irreversible change occurring in the ionized form of the enzyme (if it be an acid) or in the unionized form (if it be a base).

3. A further progressive autodestruction of pepsin at any given hydrogen ion concentration and temperature is defined by the mass law equation for a monomolecular reaction

4. The velocity of autodestruction of pepsin is directly proportional to the hydroxyl ion concentration. It is much less in the range of hydroxyl ion concentration from pOH 9.89–7.7, than in the range greater than pOH 7.7. In both of these ranges variations in pK with pOH may be represented by straight lines.

The authors wish to thank Mr. M. H. Centner for his generous assistance with numerous analyses.

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THE EFFECT OF CERTAIN RESPIRATORY INHIBITORS ON THE RESPIRATION OF CHLORELLA.

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INTRODUCTORY REMARKS.

This paper presents experiments on the effect of hydrocyanic acid, hydrogen sulfide, and carbon monoxide on the respiration of the green alga, *Chlorella*. The subject is of interest because all three of these substances are known to inhibit respiration specifically and reversibly in various organisms.

Hyman,[1] in a paper on the effects of potassium cyanide on *Planaria*, has reviewed the cases where direct measurements of the effects of cyanides on respiration have been made. Negelein[2] has published experiments on the effects of hydrogen sulfide, and Warburg[3] on the effects of carbon monoxide.

It has been shown in this laboratory that these three inhibitors of respiration do not check the respiration of *Chlorella*. Warburg[4] has shown this for hydrocyanic acid, Negelein[2] for hydrogen sulfide; I have tried the effect of carbon monoxide.

As will be shown, this exceptional behavior of *Chlorella* vanishes when the alga is made heterotrophic.

Methods.

The methods used in this work were essentially the same as those of Warburg and Negelein in their work on *Chlorella*. The alga was cultivated as described by them,[5] in a water thermostat lighted continuously with three 75-watt metal filament lamps, about 30 cm distant from the culture flasks. A slow stream of 5 per cent carbon dioxide in air was bubbled through the cultures. They were pure for the most part, but in control experiments with cells from cultures where no precautions were taken to exclude bacteria the same results were obtained as with cells from cultures known to be pure. The amount of *Chlorella* cells used so far exceeded that of any chance bacteria, that the latter did not affect the results.

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[1] Hyman, L. H., *Am. J. Physiol.*, 1919, xlviii, 340.

[2] Negelein, E., *Biochem. Z.*, 1925, clxv, 203.

[3] Warburg, O., *Biochem. Z.*, 1926, clxxvii, 471.

[4] Warburg, O, *Biochem. Z*, 1919, c, 268.

[5] Warburg, O, and Negelein, E., *Z. physik. Chem*, 1922, cii, 250.

Respiration was measured manometrically, in the dark. Measured quantities of cell suspension were pipetted into vessels of the type shown in Fig. 1. The vessels were connected by gas-tight joints with their respective manometers. The gas space above the cells, and the capillaries, as far as the manometric fluid, were filled with a mixture of 5 volumes per cent carbon dioxide in air before the system was closed The vessels were shaken in a water thermostat at 20°C in order to maintain a state of equilibrium between the gas in the space above the cell suspension and that dissolved in the suspension. It was always fast enough so that an increase caused no change in the results. At intervals the shaking was interrupted for the purpose of reading the manometers.

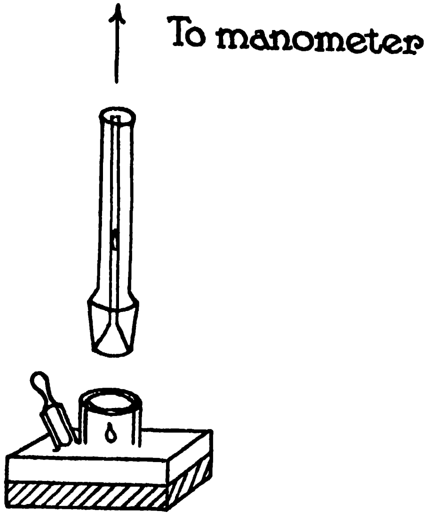

FIG. 1

The principle involved here is that oxygen is less soluble in the cell suspension than carbon dioxide. Thus, if the cells, in respiration, consume oxygen and give off the same amount of carbon dioxide, the pressure in the gas chamber will decrease, causing a change in the manometer (*cf.* Warburg[6] for a detailed discussion of this method).

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[6] Warburg, O., *Biochem. Z.*, 1924, clii, 51.

Experiments with Hydrocyanic Acid.

The experiments with hydrocyanic acid will be described first and most completely. They are typical of those performed with hydrogen sulfide and carbon monoxide.

The concentration of hydrocyanic acid was 10^{-4} normal in practically all experiments. This is a convenient concentration for purposes of comparison, as Negelein has used it also. Its effect on ordinary *Chlorella* cells (*i.e.* cells grown in an inorganic medium in bright light), is shown by the two lower curves of Fig. 2. Respiration is slightly accelerated. The two upper curves of Fig. 2 show the effect of the same concentration of hydrocyanic acid on the respiration of similar cells suspended in a solution containing 1 per cent glucose. It reduces their respiration over 50 per cent. Since all four curves represent the oxygen consumptions of the same amounts of cells, a com-

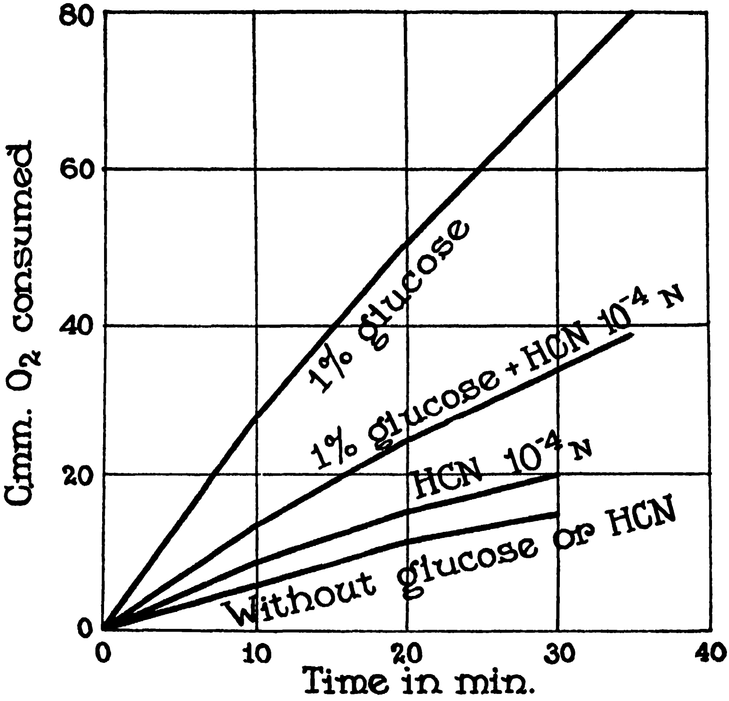

FIG 2.

parison of the top and bottom curves shows that 1 per cent glucose about quadruples respiration.

Osterhout[7] and Krehan[8] have shown that hydrocyanic acid has definite effects on the permeability of living cells. Hence it might be argued that the acid merely checks the penetration of the sugar into the cells, and not the oxidation process inside them. It may be shown in various ways that this is not the case.

Cells may be allowed to remain in a medium containing sugar for some time before the addition of hydrocyanic acid. When it is thus later added, the resulting inhibition is the same as when it is added with the sugar.

Another method is to grow *Chlorella* in a medium containing 1 per cent glucose. For the experiment, the cells are centrifuged off and transferred to an inorganic medium. The respiration of such cells is checked 40 to 50 per cent by 10^{-4} normal hydrocyanic acid.

The most conclusive method is to take autotrophic cells, grown in an inorganic medium, allow them to stand in 1 per cent glucose solution until the sugar has had time to penetrate (about 15 minutes), and then return them to the inorganic medium in order to measure the respiration and the effect of hydrocyanic acid. Respiration is reduced 60 to 70 per cent.

Organic substances of various sorts other than glucose have been tried, to see whether they would render *Chlorella* respiration sensitive to hydrocyanic acid. Those which, like glucose, call forth a respiration of three to four times the normal, were used successfully. Checking by hydrocyanic acid was the same as in glucose. Such substances are fructose, galactose, and mannose. All act almost exactly alike. The effect of the sugar on the respiration is very independent of the concentration. Glucose, for example, accelerates the respiration about four times, whether it is present in 4/100 per cent or 4 per cent solution. The inhibition by hydrocyanic acid is likewise independent of the sugar concentration.

Other substances tried gave slight acceleration of respiration (in general less than double). Many indifferent substances will cause this slight acceleration. But no real sensitivity to hydrocyanic acid results. Although respiration may be checked slightly at first, it is soon accelerated, just as with ordinary cells. The substances tried were cane-sugar, arabinose, dioxyacetone, glycocoll, mannitol, and lactic acid. Fig. 3 shows the effect of hydrocyanic acid on cells suspended in a solution containing 1 per cent glycocoll. Glycocoll was selected as typical of the indifferent substances.

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[7] Osterhout, W J. V., *Bot. Gaz*, 1917, lxiii, 77.

[8] Krehan, M., *Internat. Z. physik.-chem. Biol.*, 1914, i, 189.

Various authors (*e.g.* Hyman[1]) have stressed the reversibility of the hydrocyanic acid inhibition. If the effect be irreversible, it is argued that the cells have been injured, and any inhibition of respiration cannot be regarded as specific. To test the reversibility in the case of sugar-containing *Chlorella* cells, the cells were subjected to the effect of hydrocyanic acid for 30 minutes, the checking of respiration measured, and then a stream of moist air was bubbled through the suspension, to remove the hydrocyanic acid. Respiration was then measured again, and found to be greater than in the control. The effect is therefore completely reversible.

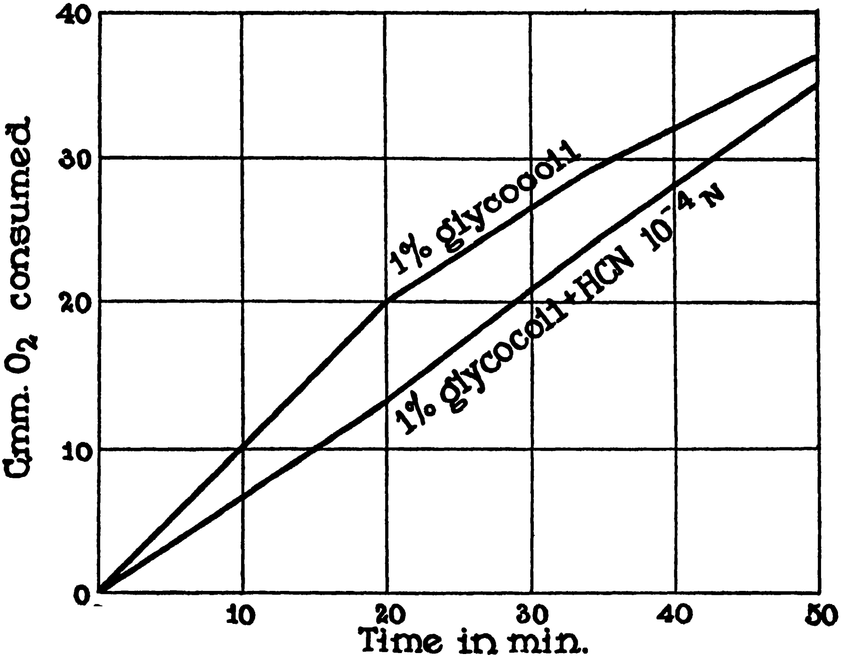

FIG 3.

Experiments with Hydrogen Sulfide.

Similar experiments were carried out with hydrogen sulfide and led to the same results as with hydrocyanic acid. Free hydrogen sulfide was formed in the cell suspension by the addition of an appropriate amount of N/10 solution of sodium sulfide. The solution always contained acid phosphate which reacts with Na_2S to form free H_2S. (For the calculation of the correct amount of sodium sulfide to add in order to achieve a given concentration of hydrogen sulfide in the solution, see Negelein's paper on the effects of hydrogen sulfide.) Equilibrium between solution and the gas chamber is established, a large amount of hydrogen sulfide remaining in the gas chamber.

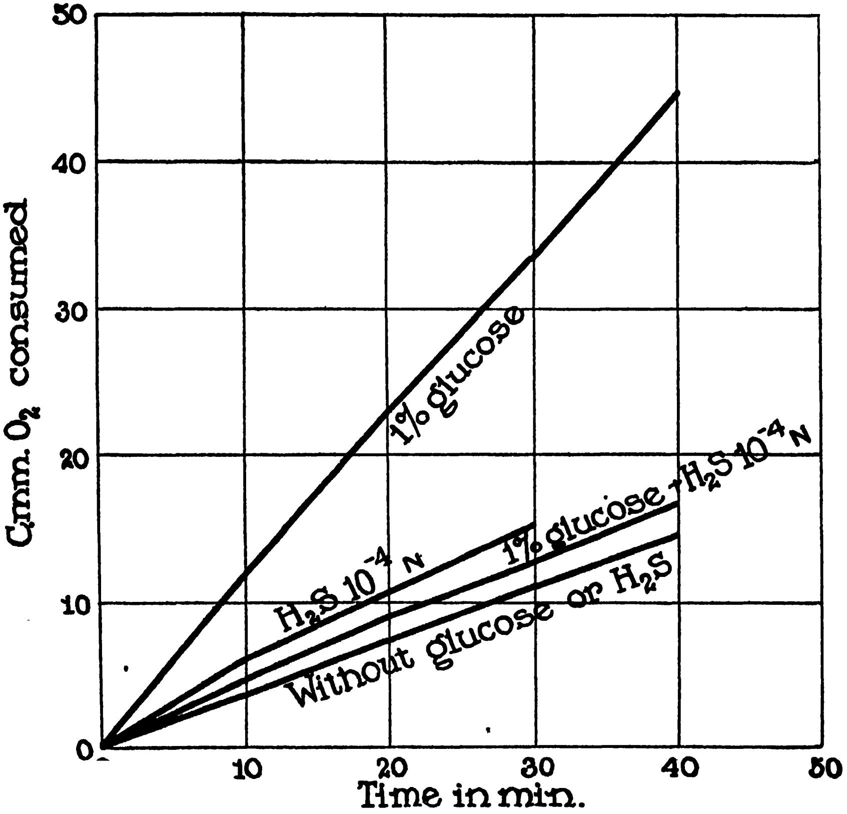

FIG. 4.

Fig. 4 shows the effect of hydrogen sulfide on ordinary *Chlorella* cells and on cells suspended in 1 per cent glucose solution. It is essentially the same as Fig. 1, which shows the corresponding curves for hydrocyanic acid.

The hydrogen sulfide effect was tested for reversibility, and it was found, like that of hydrocyanic acid, to be completely reversible. After removal of the hydrogen sulfide, respiration was greater than before it had been added.

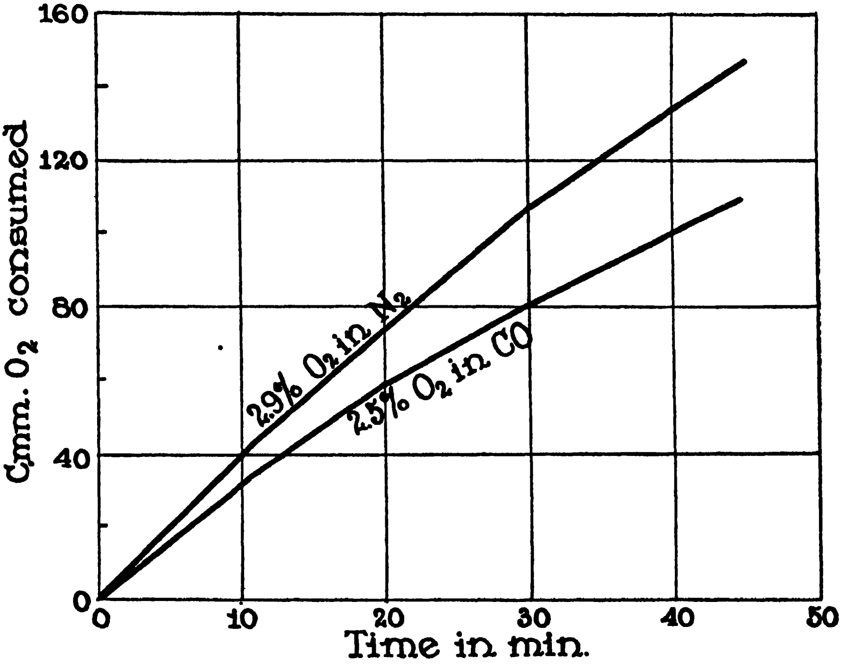

Fig. 5.

Experiments with Carbon Monoxide.

In the experiments with carbon monoxide, a mixture of oxygen and carbon monoxide replaced the usual 5 per cent carbon dioxide in air used in the gas space. As control the same amount of oxygen in nitrogen was used. Fig. 5 shows the effect of a mixture of approximately 2.5 volumes per cent oxygen in carbon monoxide on *Chlorella* suspended in a solution containing 1 per cent glucose. No curves are given for the effect of carbon monoxide on ordinary *Chlorella* cells, for it does not affect their respiration.

The inhibition of respiration by carbon monoxide is completely reversible. Carbon monoxide was removed by passing the oxygen-nitrogen mixture through the gas chamber.

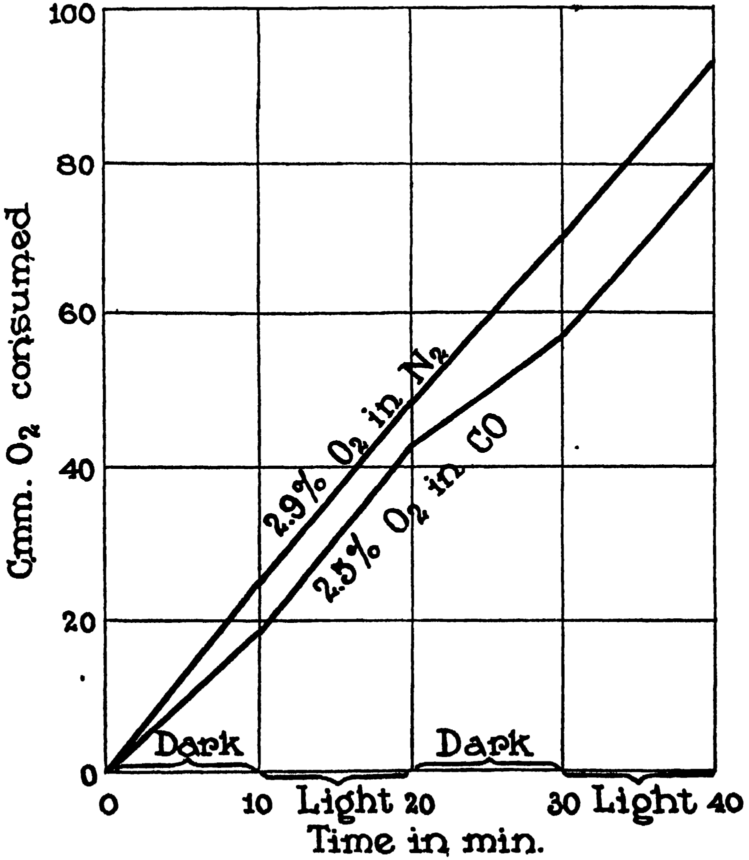

Fig. 6.

Warburg[3] has shown that the inhibition of yeast respiration by carbon monoxide practically vanishes in light. This experiment cannot be performed with ordinary *Chlorella*, as photosynthesis takes place in the light. But yellow non-photosynthesizing *Chlorella*, practically chlorophyll-free, may be produced in a medium containing

1 per cent glucose and of low iron content. Such cells are completely heterotrophic, and their respiration is inhibited by hydrocyanic acid and hydrogen sulfide. Respiration is practically the same in light as in darkness. Fig. 6 shows the effect of successive periods of light and darkness on the respiration of such cells in 2.5 per cent oxygen in carbon monoxide, and in 2.9 per cent oxygen in nitrogen.

SUMMARY.

Chlorella, when made heterotrophic by means of certain sugars, respires like other heterotrophic cells when subjected to the respiratory inhibitors, hydrocyanic acid, hydrogen sulfide, and carbon monoxide.

Whether the case of *Chlorella* is typical for green cells in general remains to be seen. Experiments with various other green organisms are being carried out, in hope of settling this point.

My thanks are due to Professor Otto Warburg for suggestion and criticism during this work.

THERMAL INCREMENTS FOR PULSATION-FREQUENCY IN "ACCESSORY HEARTS" OF NOTONECTA.

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(Accepted for publication, January 20, 1927.)

I.

For development of a theory of the temperature characteristics of vital processes it is necessary to deal with types of material in which it may be expected that experimental treatments can evoke one or another of a series of interrelated critical increments (Crozier, 1924–25, *a*, *b*; Crozier and Stier, 1924–25, *a*; 1925–26, *b*). In this paper we give an initial account of temperature relations in a kind of rhythmic activity which is in certain respects especially favorable for such tests.

Pulsatile organs, "accessory hearts," were first observed in the legs of juvenile aquatic hemiptera (*Notonecta*, *Ranatra*, *Corixa*, and others) by Behn (1835). These organs, located generally in the tibia just distal to the femoral articulation, or in the tarsus, were conceived by Behn to be a kind of "membranous valve" important in maintaining the peripheral circulation. Their "spontaneous" rhythmic movements were apparently confirmed, although misinterpreted, by Dufour (1835); they were discussed by Dugès (1838[1]), and by Verloren (1847[2]), and a summary of the early observations on the "accessory hearts" in aquatic and other hemiptera was given by Edwards (1858). It was noted (Verloren, 1847) that in *Tettigonia* the pulsatile activity might be interrupted for more or less lengthy periods, but that when active the frequency of the quick, sharp contractions exceeded that of the heart. The "hearts" were rediscovered by Mitchell (1858). Locy (1884) also described their movements, in *Ranatra*, *Notonecta*, and *Bdelostoma*, subscribing to their rôle in connection with the circulation; he noted that the movements would continue for a time in amputated legs, and even when the "heart" was itself cut into parts. More recent observations, with figures, are given by Brôcher (1909). The existence of these organs is briefly referred to in entomological texts (Houlbert, 1920; Schröder, 1913), but nothing further appears to be known about these curiously inviting instances of perhaps myogenic rhythm. Among a number of possible sources of pulsatile organs in the bodies of insects (*cf.*, *e.g.*, Brôcher, 1916; 1917; 1919) they are by far the most amenable to experimental manipulation. They can be observed in the intact animal, and proper technique provides preparations in which the organs are active for days in detached legs. Six preparations may be gotten from a single individual, permitting for some purposes unusual control material.

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[1] Dugès, 1838, p. 441.

[2] Verloren, 1847, p. 82.

In our experience the locomotor muscles of amputated legs of *Corixa* are likely to exhibit twitching movements, which interfere with the activities of the "accessory hearts," and in *Ranatra* the latter are difficult to see. Common species of *Notonecta*, however, are obtainable in large numbers, live very well in the laboratory, the "hearts" are easily seen, and the limb muscles of the detached legs are absolutely quiescent. The following account is based entirely upon the behavior of "accessory hearts" in the two swimming-legs of adult *Notonecta undulata*. It will be shown that although two preparations are obtainable from each individual these may nevertheless behave in quantitatively different ways, so that perfect control observations are not possible. But no necessity for such controls arose in the present work, which was designed to obtain primarily the temperature characteristics for pulsation-frequency during the survival of the isolated limb.

Our purpose was to discover if in different freshly isolated legs, and during the course of the irreversible death phenomena, there should not appear, as in the breathing movements of grasshoppers (Crozier and Stier, 1924–25, *a*), a series of differing critical thermal increments for pulsation-frequency, which might thus reveal certain of the interrelated processes involved in determining the rate of pulsation.

We were especially anxious to do this because of the possibility of obtaining information about typical governing processes in insect muscle, for comparison with central nervous activities (Crozier, 1924–25, *a*; Crozier and Federighi, 1924–25, *a*, *b*; Crozier and Stier, 1924–25, *a*, *b*; Fries, 1926–27). Although possibly containing nervous elements, the "hearts" in isolated legs are obviously beyond central nervous influence. It turns out that the typical temperature characteristics obtained for frequency of pulsation agree quantitatively with those known for respiratory phenomena (Crozier, 1924–25, *b*), and for breathing movements of insects (Crozier and Stier, 1924–25, *a*), but with the addition of a further member of this "set" of critical increments. They differ sharply from the value (μ = 12,300) which is characteristic for frequency of heart contractions in arthropods (Crozier, 1924–25, *a*; Crozier and Federighi, 1924–25, *a*; Crozier and Stier, 1925–26, *b*; Fries, 1926–27). The associations observed between the four chief increments obtained are of additional interest for the analysis of vital processes from this standpoint.

II.

The beating of the leg-"hearts" is easily observed in the intact animal. The back-swimming habit of *Notonecta* makes it possible to prepare an individual by mounting it, ventral surface upward, upon a small block of hard rubber, to which it is attached by a small amount of vaseline, in such a way as to be at the surface of a vessel of water, with the legs outstretched in the surface film. Occasionally, movements of the appendages are seen; but the intervals between these more or less periodic movements are fairly long. The rate of pulsation in any one leg is very uniform; thus in one case, observed at frequent intervals over 24 hours, at 17°C., the time for ten contractions varied between 9.6 and 1.06 seconds; in another, between 5.2 and 5.6 seconds during 36 hours; frequently there is some decline in rate after 12 hours.

The beats are irregular for brief periods; sometimes a contraction is skipped. There is no evidence of diurnal rhythm. When the two swimming-legs of one individual are compared, there is usually a distinct difference between them:

Animal No.	Leg	Time for ten pulsations, seconds								
N1	Left	5.4	5.6	5.3	5.5	5.5				
19°	Right	7.1	6.7	7.3	7.3	7.8	8.0			
N2	Left	5.0	5.1	5.1	4.7	4.1	5.2	4.9	5.8	5.0
19°	Right	4.4	4.9	4.7	5.6	5.1	5.5	5.7	4.4	
N3	Left	8.3	7.9	7.9	8.3					
17°	Right	7.4	7.0	7.6	7.0					

One leg-"heart" may abruptly cease operations for a time, the other continuing without pause. When the animal is stimulated, the beat may be hastened or retarded and irregularities appear.

The uncorrelated nature of the rates of contraction in the two legs and their independent variation speak for local rather than central nervous control of the activities of the leg-"hearts." Yet when the intact animal dies the frequency of the pulsations changes and to some extent their character also. When an animal in which the "heart" of one leg has been pulsating for some time at a steady frequency of 10.5 seconds for ten beats is completely covered by a thick layer of vaseline the rate remains constant for a time; but then, after several hours, the rate *suddenly* decreases to about one-half its former value, in both legs, and at this point the animal as a whole ceases to respond to stimulation. If left attached to the body the leg-"hearts" continue to beat for some 14 hours, but with decreasing although regular frequency. If detached from the body the legs show sustained pulsation for a much longer time. If the attached or isolated limb be swathed in vaseline no change in pulsation rate occurs.

These and other observations show that the rate of pulsation is locally determined, but that a secondary control, of nervous character, possibly, or dependent upon changes in the hemolymph pressure, is also important.

Two general types of result follow amputation of a swimming-leg. The rate of pulsation of the "heart" may remain the same as before amputation, the beat becoming at once more regular, and then, after about an hour (18°), the beat suddenly becomes much slower. In other cases the rate immediately after amputation may be about doubled, for several minutes, followed by sharp cessation of contraction which lasts about an hour; contractions are then resumed at about one-quarter of the original frequency. At the moment of pinching the leg with scissors the beats slow down, then become very rapid, then settle down to the initial rate. We have plugged the ends of amputated legs by exposing them to the air for 5 minutes, then by coating with vaseline; or the leg may be cut while imbedded in vaseline. No effects of these procedures were detectable.

It is important that the nature of the pulsatile movement of the leg-"heart" retains a very uniform character throughout the temperature range. Contraction is abrupt, relaxation slower, followed by an interval of quiescence. The contraction is maximal and always complete. The sheet of muscle fibers constituting the "heart" (*cf.* Brôcher, 1909) is cap-shaped at the proximal end, from which a (?muscle) band passes to the femorotibial joint; distally, the "cap" spreads out into a flat contractile band. As a rule, contraction first appears at the apex of the "cap," and travels as a wave to the distal band. Occasionally the contraction wave is reversed. As death approaches the relaxation phase is prolonged, and the quiescent interval almost disappears; this also happens if the leg is sealed in a tube of water previously boiled.

III.

For observation, the isolated legs of *Notonecta* were attached by vaseline to a glass slide placed in a small glass vessel of water. This vessel, having a flat bottom, was sunk in a chamber filled with water. The chamber was water-tight, and addition of hot or cold water for regulation of temperature was by way of a coil of copper tube, with many small apertures, connected with an external supply. This chamber was immersed in a large stirred thermostat. Through the cover of the vessel containing the preparations the objective of the observing microscope passed, and also a thermometer reading to 0.01°. The objective was used as a water-immersion lens, thus avoiding troublesome fogging, all the metal surfaces being coated with paraffin to obviate oligodynamic effects. The regulation of the temperature of the innermost vessel was secured by maintaining very slight differences between the temperature of the large thermostat and of the chamber immediately surrounding the observing vessel. Two, three, or four legs could be mounted together, and by adjustment of the microscope they could be viewed in succession or as desired. Light was reflected up through the thermostat and through a window in the inner box.

The muscles of an isolated leg, including the contractile fibers of its "heart," must of course be regarded as a system proceeding toward death, irreversibly. Therefore, if the relationship is to be obtained which exists between temperature and frequency of pulsation it is necessary to work rapidly in order to secure observations at a number of temperatures before the underlying mechanism shall have changed materially. With these preparations it is possible to do so, largely because the latitude of variation in frequency of pulsations at constant temperature is so very slight.

After removal from the animal about 1 hour (at room temperature) is often necessary before the isolated "accessory heart" settles down to a steady rate of contraction. Not infrequently, however, this steady rate, lower than before amputation of the leg, is attained almost at once. A few successive readings of time for ten pulsations enable one to judge if the preparation is in a state suitable for experimentation. In order to discover the degree of constancy to be expected under uniform temperature, graphical records were obtained with the aid of a signal key and a chronoscope registering 1.0 second intervals upon a smoked drum. In this way a sufficiently precise record was obtained of continuous series of pulsations over periods of about ½ hour. The high degree of constancy in "time for ten pulsations" in any one preparation is shown in the following table:

Temperature	Preparation	Mean time for ten contractions
°C.		*sec.*
16 4°	A_1	19 0 ±0 4
16 4°	B_2	21 6 ±0 2
16 4°	E_2	19 2 ±0 3
16.1°	I	17 95±0 95
16 2°	K	15.7 ±0 4

In this table the ± limits give the *maximum* latitudes of variation, which average 4.9 per cent of the means. The same type of variation is evident in observations made at intervals, with a stop-watch, over periods of some hours. The maximum latitude of variation for any one heart is not over 10 per cent, and is as a rule less than 5 per cent of the mean at a given temperature. This variation of course includes errors of observation. The same type of fluctuation is apparent throughout the temperature graphs. In good preparations constancy in pulsation rate is maintained for many hours, at uniform temperature.

IV.

When the temperature is varied it is quite necessary to study each preparation individually. Averaging observations from different "accessory hearts" is not permissible. Even the two swimming-legs from the same individul may differ significantly (Figs. 7, 8), although they not infrequently give parallel results (Figs. 1 to 5).

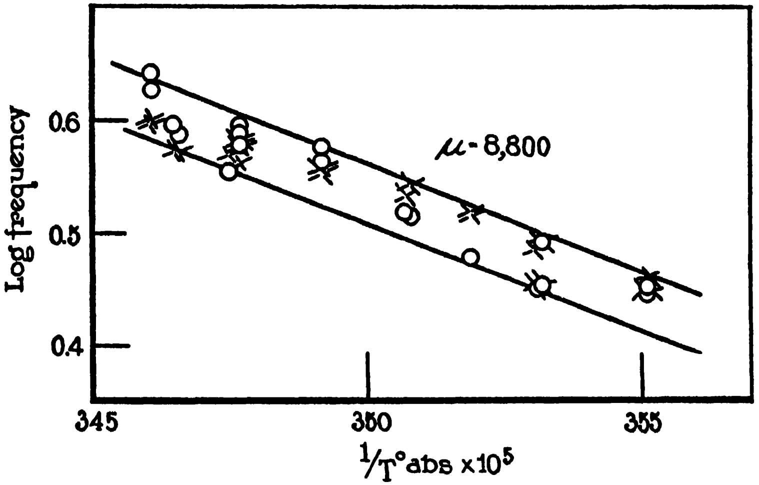

FIG. 1. In this and succeeding figures the temperature characteristics for frequency of pulsation in the accessory leg-"hearts" of *Notonecta* are obtained graphically by plotting *log frequency* (= log [100/seconds for 10 beats]) against *reciprocal of absolute temperature.*

Preparations Nos. 29 (circles) and 30 (crosses), (the latter having the rates multiplied by 2.0 for comparison) give $\mu = 8,800$. It may be noted that the latitude of variation, with the large scale-units employed, corresponds to an extreme difference, at the left end of the graph, of only 1.4 seconds in a mean of 23.4 seconds for ten pulsations.

In this way we have examined a large number of preparations, of which about 30 were studied in detail. The total number of observations was above 3,000. Since we desired especially to know the kinds of critical increments which might appear during the onset of death, it was necessary to avoid so far as possible the production of irreversible effects by exposures to very low or to very high temperatures. It was also necessary to work rapidly in order to make sure of time in

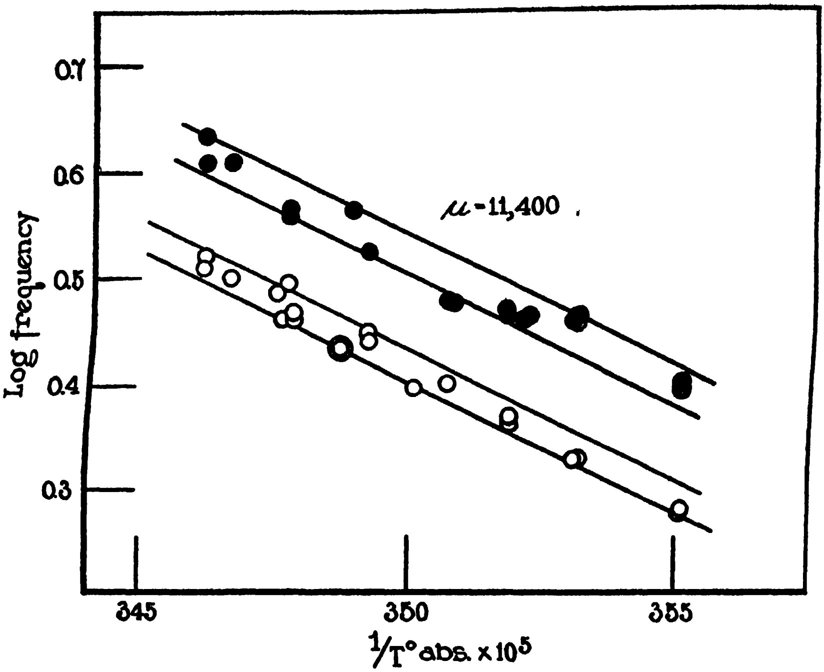

FIG. 2. Legs Nos. 27 (open circles) and 28, from the same individual, give μ = 11,400

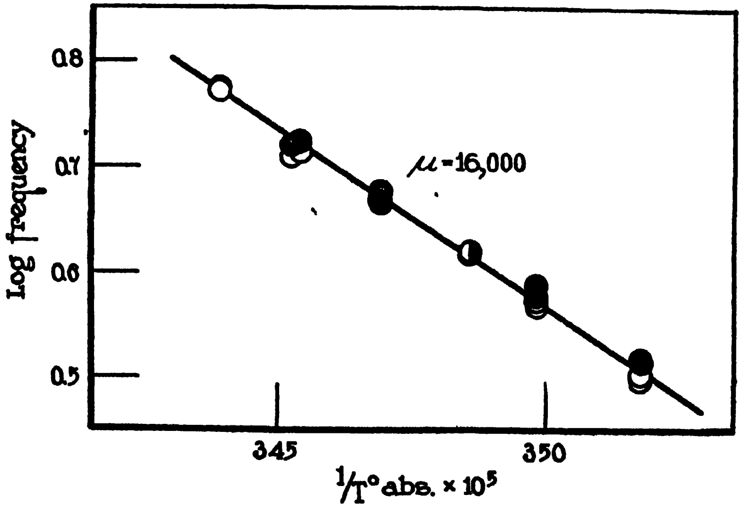

FIG. 3. Legs Nos. 19 and 20 give sensibly identical results; μ = 16,000.

which to travel in reverse order the excursions up or down the temperature range before intrinsic change of temperature characteristic should supervene. The absence of great natural variation in rhythm, coupled with the non-interference of spontaneous activities of the leg musculature, greatly facilitated such observations. The agreements in values of μ obtained show that confusion from these sources was successfully avoided.

The lower critical temperature for continued pulsation was 5°; in the isolated legs 20° was found a very definite upper limit for regular rhythm. In a number of preparations pulsations ceased at 9.5° ± 1.0°. The intermediate temperatures at which "breaks" appeared, indicating change of increment or of frequency, varied from 10.8° to 17.2°; the reason for this variation, as subsequently discussed, is found in the progressive exhaustion of the pulsating system, with induction of physicochemical changes independently of the thermal ones.

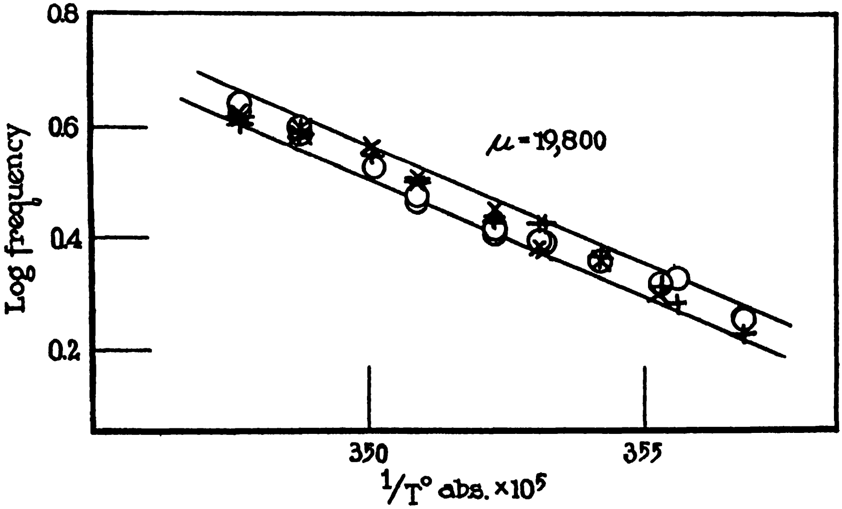

FIG. 4. Three preparations, Nos. 50, 51, and 52, give μ = 19,800. (The rates for No. 51 have been multiplied by 1.102, and those for No. 52 by 1.026.) These were from different individuals.

V.

The results of these observations are sufficiently illustrated by the examples given in Figs. 1 to 11. Including those instances in which "breaks" occurred, as exemplified in Figs. 6, 8, and 11, the following is a summary of the critical increments found to describe the change in frequency of contraction as determined by temperature.

Mean μ	Extreme range	Number of instances
8,190	7,900 to 9,150	14
11,350	11,290 to 11,500	4
16,200	15,100 to 17,000	16
19,800	19,600 to 20,000	4
24,530	23,000 to 25,000	5
32,200	30,000 to 34,000	12

The most precise determinations of μ are of course only obtainable from "runs" over a good range of temperatures; since in some cases such ranges were impossible to obtain in the present experiments, a certain variation in each value of μ as ascertained is to be expected. Certain details regarding the observations are discussed in the legends of the several figures.

The other magnitudes of μ, save 32,200, have already been recognized as occurring repeatedly in connection with a variety of biological processes (Crozier, 1924–25, *b*; 1925–26, *b*). Their reappearance here adds to the conviction that they correspond to chemical realities in living matter, perhaps to the heats of activation of commonly occurring catalysts (Crozier, 1924–25, *a*).

It will be noticed at once that the most frequently occurring magnitudes are, approximately, 8,000, 16,000, and 32,000 calories. Bliss (1925–26) has described a case in which temperature characteristics of these general magnitudes apply respectively to different parts of the total temperature range as affecting the time required for a particular developmental phase in *Drosophila*. This kind of progression tempts speculation. The additive nature of heats of activation is recalled. But we believe that for the present the fact may most safely be recorded devoid of speculative fringes. This is especially so because if the individual records are examined it is found that with any one preparation there is no orderly association or succession of values such as 8,000; 16,000; 32,000 in different parts of the temperature range, or at different times. The increment 16,000, for example, may be associated with 32,000, or with 8,000; or 8,000 with 32,000. This speaks for a certain random association of the processes manifested by the appearance of the respective increments; with this restriction, however, the lower of two increments always pertains to the higher temperature range.

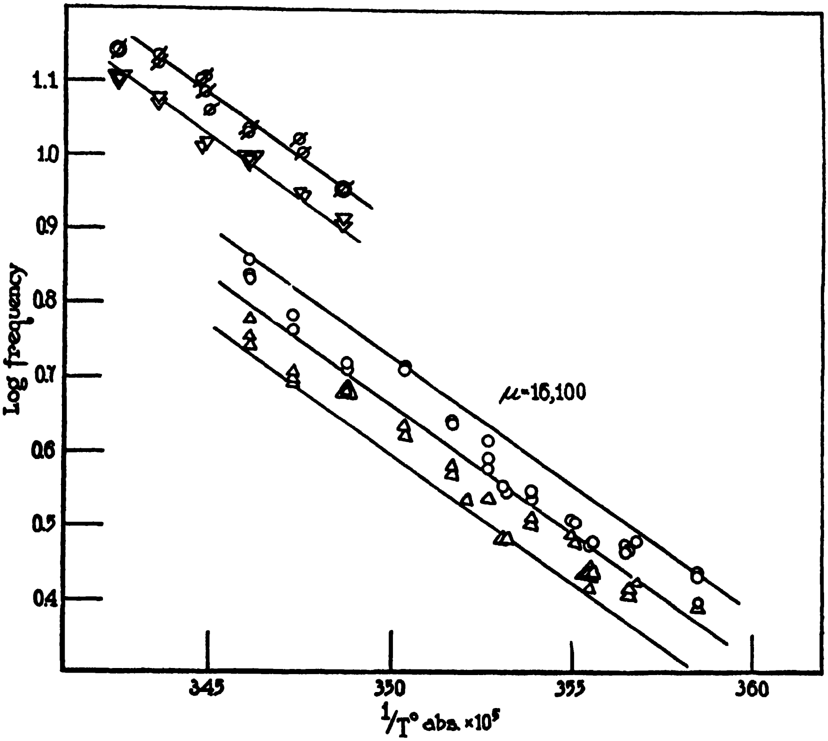

FIG. 5. Leg-"hearts" Nos. 31 and 32, from the same individual, give $\mu = 16{,}100$. The rate changed markedly after 24 hours, without noticeable change of μ, and by about the same amount in each leg.

In an isolated and irreversibly deteriorating system such as is presented by the isolated leg-"heart" it is to be assumed that changes in μ should also be evident as a function of time.

By "a change which is a function of time" we mean to imply that the processes of exhaustion and death should influence the occurrence of "breaks" in the relation between pulsation-frequency and temperature. The simplest illustration of such an effect is given by cases such as that shown in Figs. 5 and 8. It is obvious that the differential effects of exhaustion upon each of several processes such as might possibly control the frequency of pulsation may result in these elements of the nexus governing pulsation being successively revealed as determining the relationship to temperature. In numerous cases

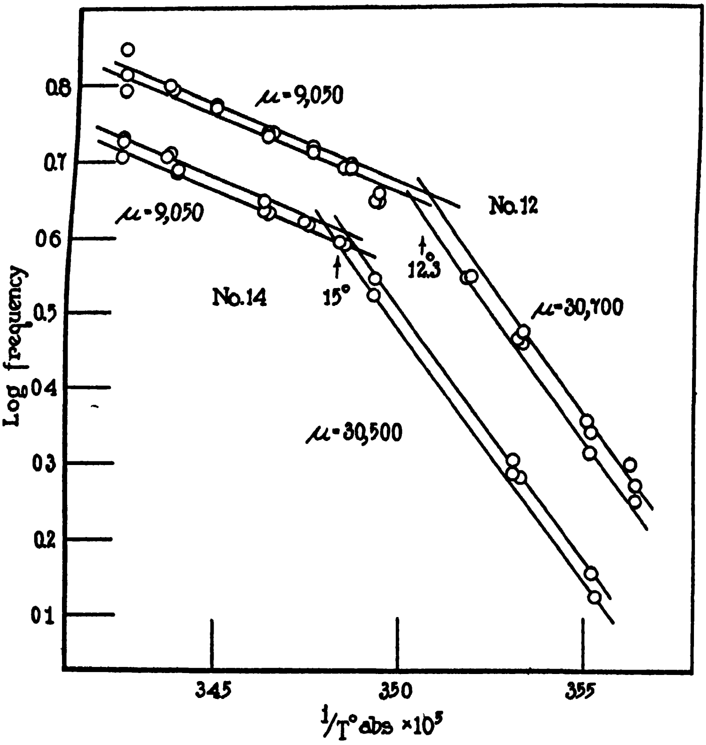

Fig. 6. Two preparations from different individuals, show transitions from $\mu = 9{,}050$ to $\mu = 30{,}700$ at lower temperatures.

where this sort of result is evident, reversibly, on passing from one zone of temperatures to another (Crozier, 1924–25, *b;* 1925–26, *b*) it has been assumed that it may be due to the fact that a catenary chain of reactions underlies the determination of frequency of pulsation (Crozier, 1924–25, *b*); the fact that the temperatures at which these shifts occur are not distributed at random (Crozier, 1925–26, *a*) makes it necessary to suppose that physical changes, essentially of a grossly discontinuous character, are also implicated. In dealing with the *Notonecta* leg-"hearts" it was expected that the degradation of the excised system would have similar effects, and that in consequence the occurrence of an intermediate critical temperature should be blurred. This is the fact, but there is ample evidence, nevertheless, that in the neighborhood of 15° (10.8° to 17.2°) abrupt changes most frequently occur in the relationship between rate and temperature.

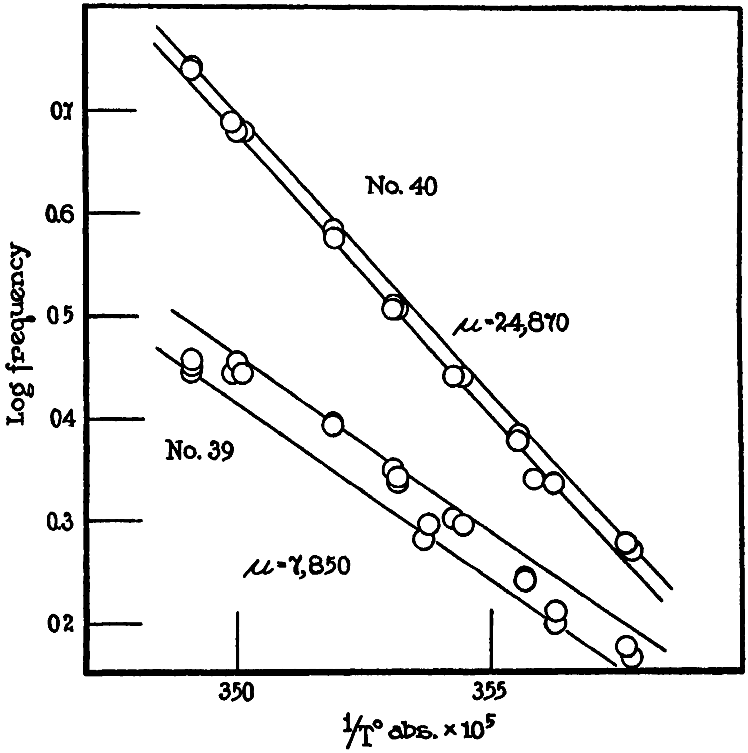

FIG. 7. Two leg-"hearts" (Nos. 39 and 40) from one individual give, respectively, μ = 7,900 and μ = 24,900. (The rates for one (No. 39) have been divided by a factor, 1 4, to permit unconfused plotting)

A further sort of complication in these curves is due to what we have characterized in other instances (Crozier and Stier, 1925–26, *a*) as a change of frequency or velocity without change of increment, or, what is probably connected therewith in an intimate way (Crozier and Stier, 1925–26, *b*), a change in the latitude of variation at constant temperature without change of increment. This type of effect occurs in certain *Notonecta* preparations (Figs. 9, 10, 11) and might easily confuse interpretation. To what extent these shifts may be due to change of pace-making location in the pulsating organ we cannot decide; in most instances, we feel, this explanation would not be correct.

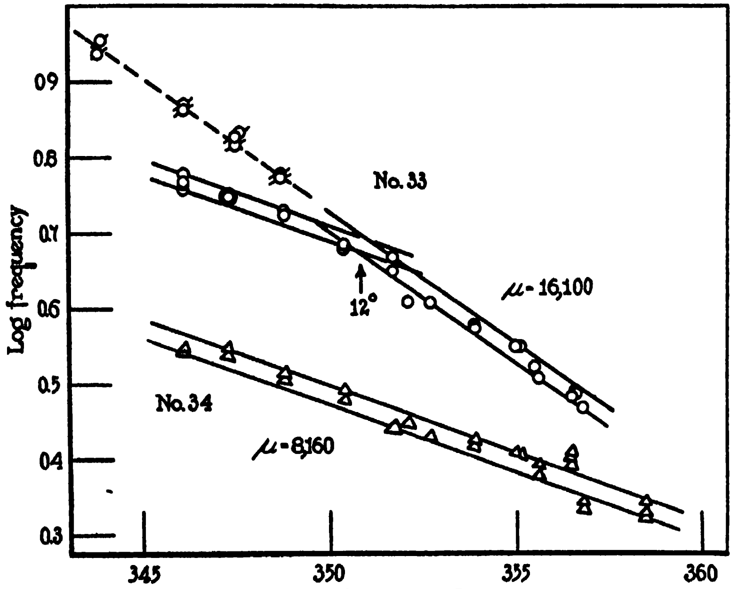

FIG. 8. Two preparations from one individual give, in one case $\mu = 8,200$, in the other $\mu = 8,200$ above 12°, 16,100 below 12°; on reversing the course of the temperature changes (dashed line), next day, the increment 16,100 is now found to hold in this case above 12°. This type of change is one of those presumed to depend upon the irreversible progress of exhaustion in the isolated legs.

VI.

The results of these experiments have been considered in terms of regularities in the nature of the influence of temperature on the frequency of pulsation in the leg-"hearts" and in a wide variety of other objects. The magnitudes of the Arrhenius constant are not distributed at random. For this the explanation has been proposed that constants E or μ serve to characterize catalytic reactions governing the frequency of pulsation. It is possible to suggest a number of reasons why this sort of regularity "ought" not to be found. One method of dealing with the direct observations is to invent empirical formulæ for their expression. One such has recently been proposed by Bělehrădek (1926, *a*, *b*) in the form $y = a/x^b$, where x = temperature

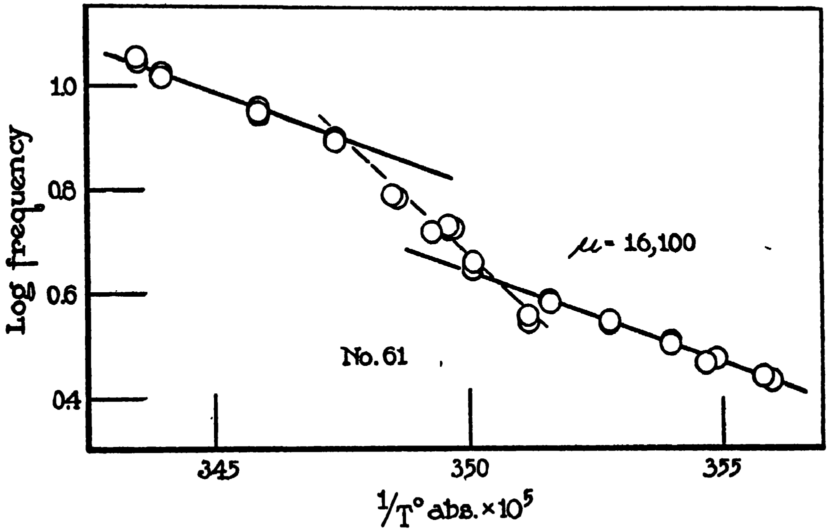

FIG. 9. A "break" in the curve of temperature relations was found in four cases, one of which is here shown, which is similar to that earlier described by Crozier and Stier (1924–25,*b*) in connection with the breathing rhythm of Anurans. The course of the observations was exactly reversed on raising the temperature. (The slope of the fitted lines (μ = 16,100) is the average of those fitting the upper and the lower segments.)

(*Centigrade*) and y = time necessary for a given phenomenon, and a and b are constants. This is obviously the well known empirical formula of Esson (Harcourt and Esson, 1895; Harcourt, 1912) for chemical reactions, with the substitution of the Centigrade temperature for T° *abs.*; it is difficult to conceive cogent reason, theoretically, for the proposed change, which simply requires making one of the constants larger without at all improving the fit. In addition to the fact that this particular formula is of a type which can be made to describe *almost* any sort of curve not possessing too abrupt discontinuities, and that at best it has simply the status of an interpolation formula, there is the insuperable objection that it does *not* fit the facts. To illustrate this we may choose examples in which a large number of observations are available (Fig. 12). The statement (Bělehrádek, 1926, *a*) that a single simple curve can describe instances of the sort

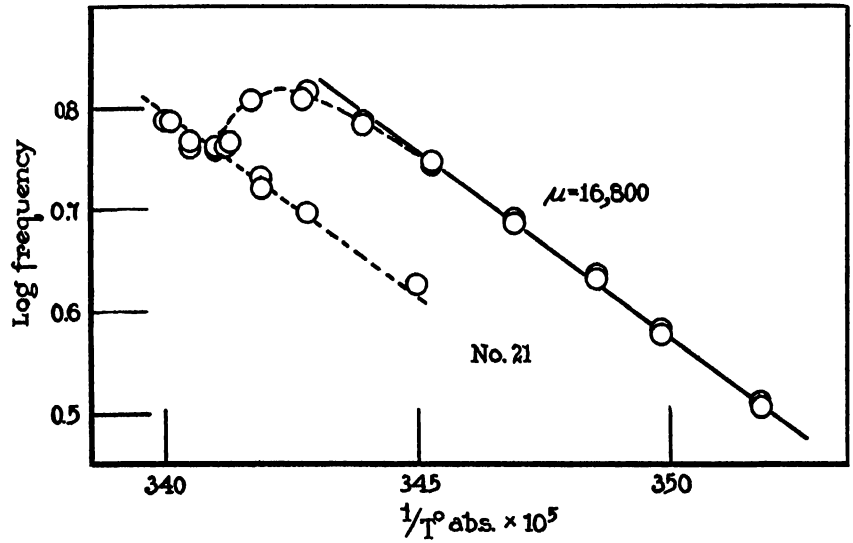

FIG. 10. In this case the temperature was first raised to a point a little above 20°; there was thus induced a permanent increase in pulsation rate, without apparent change of increment. Precisely this effect was obtained in other instances. The temperature was changed at intervals of about 15 minutes, between readings, and in the region of changing rate the points consequently fall on a curve. It is to be understood that the whole extent of vertical shift would have occurred at constant temperature.

shown in Fig. 6 is obviously futile (*cf.* Brown, 1926–27). The attempt to employ his formula has moreover led Bělehrádek (1926, *b*) into the assertion that his constant *b* reflects the primary importance of viscosity in connection with the velocities of vital processes, and that it changes systematically with the age of the organism. The notion that the temperature coefficient of a given activity decreases or increases regularly with the age of the organism is simply untrue, as our own experience with a considerable number of cases enables us to state quite definitely. We make no apology for choosing to rely upon the one type of equation which actually describes the data and which seems to stand a good chance of physical interpretation; and until some other comprehensive explanation is provided for the regularities which its use discloses we are not impressed by objections grounded, largely, on the argument that protoplasm is "too complex."[3] The efficient answer to such very general objections is, that the complexity, or rather haziness, frequently resides in the mind of the observer, and can be dissipated by increased refinement of experimental procedures.

A more interesting sort of obstacle has recently been discussed by Murray (1925–26). In his experiments the temperature characteristics deduced for frequency of pulsation in cultured explants of chick myocardium failed to show uniformity, and, in the number of preparations studied, failed to be grouped about modal values. We are not familiar with the performance of such cultures, but it can be suggested that in such a preparation, essentially an unorganized mass structurally though exhibiting regularity as to pulsation, there must exist a number of possible pace makers. It is to be expected that the net result of their fluctuating dominance might obscure the preponderating influence of any one, since it is fair to assume that their several inner metabolic states might be differently adjusted. As we have pointed out in detail in a following paper, this interpretation permits certain deductions which the figures in Murray's paper seem to us to justify.

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[3] It is perhaps of interest, since the present material enables several points of some moment to be illustrated, to comment upon an objection sometimes voiced to the practice of fitting two or even three lines to segments of such data as are plotted, for example, in Fig 6 It may be said that the implied transitions are too sharp. If one were really dealing with the effect of temperature upon a catenary series of reactions there should be a region of curvature connecting the two sensibly rectilinear zones. The answer is that in suitable instances just this indication of curvature is actually found. Most series of measurements exhibit a latitude of variation which makes it difficult to decide the precise nature of the union between the two straight lines. A number of series in the present experiments (*e.g.*, Fig. 6) do however show this sort of "rounding off" when the latitude of variation is quite small. Objections to considering the whole sweep of such series as a single curve have been mentioned in a preceding paper (Crozier and Stier, 1926–27, *a*). Sharp transitions of the sort implied in these broken graphs are of course well known in physics; we may refer to transition points, and to curves of magnetic susceptibility. Moreover, as illustrated in a recent paper from this laboratory (Brown, 1926–27), the data so fitted cannot be described by a single smooth curve when plotted directly as *velocities* against *temperature*. Again, as has been insisted previously (Crozier 1925–26, *a*), there is indication of "physical" alterations occurring at just those temperatures most frequently found to be transition points on the thermal scale. The reason for the occurrence of just these critical temperatures must be sought in the properties of protoplasm as a physicochemical system.

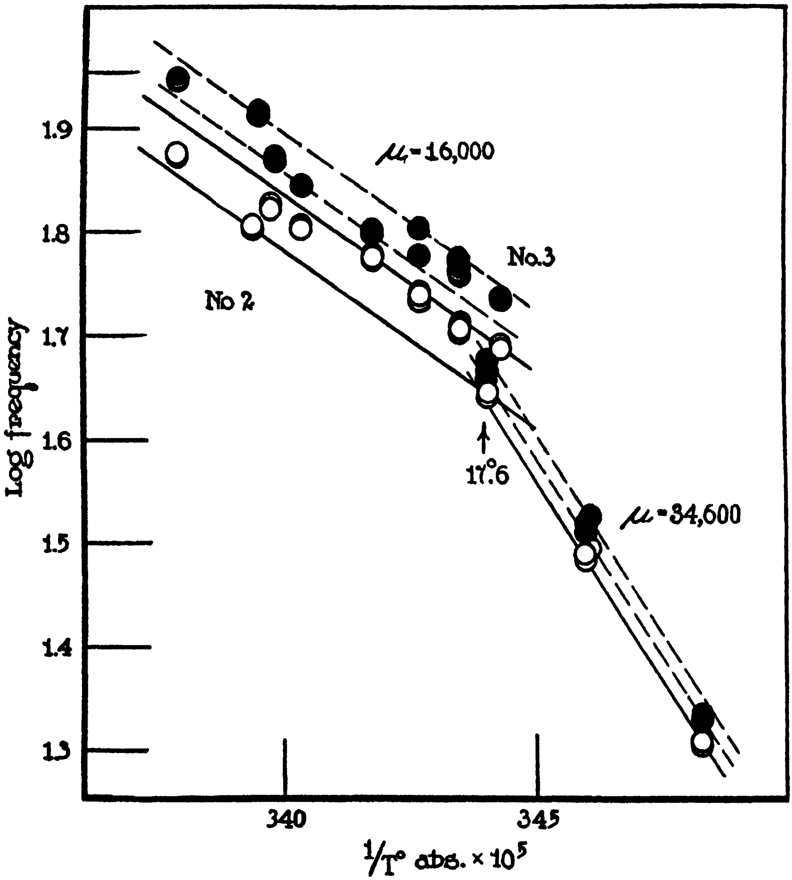

Fig. 11. Occasionally, instances were found in which an abrupt change of increment was associated with an abrupt increase of rate.

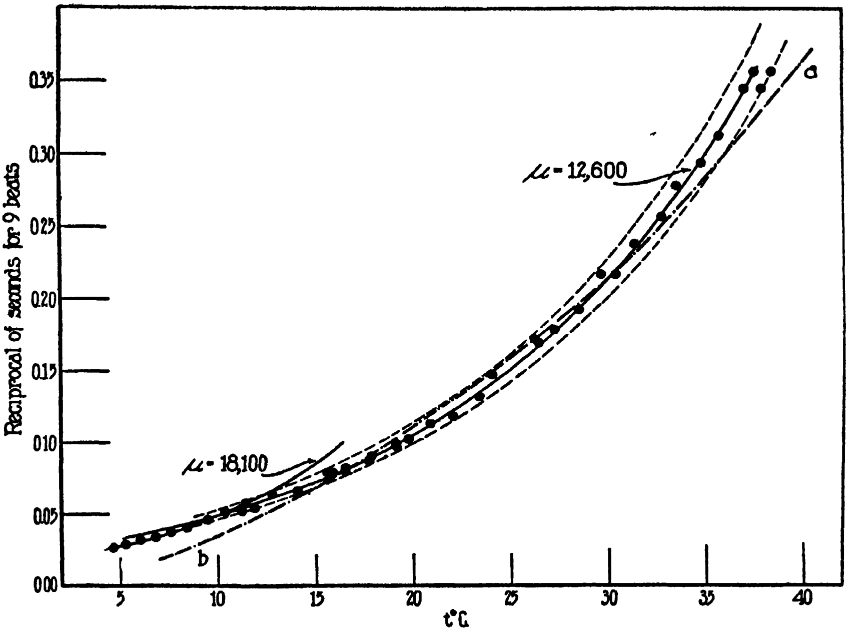

FIG. 12. Data from one experiment (in Fries, 1926–27) on the frequency of cardiac contractions in *Blatta* are fitted by the Arrhenius formula with $\mu = 12{,}600$ between 10°C. and 38°C. In the original presentation (Fries, 1926–27, Fig. 2) these data appear plotted as *log frequency vs.* $1/T°$ *abs.*, where the nature of the "break" at 10° is more clearly apparent; below 10°, $\mu = 18{,}100$. The dashed lines are transposed from the lines defining the extreme latitude of variation in the log plot (Fries, 1926–27, Fig. 2). The superiority of the logarithmic representation (*vs.* $1/T°$ abs.) consists not only in the clearer appearance of conditions on either side of a critical temperature (*e.g.*, 10° in this case), but also in the fact that the departures from the line of best fit are confined within a band the upper and lower edges of which are parallel to the central line (*cf.* Crozier and Federighi, 1925). The latter fact means that it is necessary to deal, in fitting the Arrhenius equation to such data, not with the absolute departures of the observed average rates or frequencies, but with the relative or proportionate divergences; this is of great importance if it be suggested that curves such as that in the present figure should be fitted by the method of least squares (*cf.*, also, Crozier and Federighi, 1924–25, *b*, 1925).

To curves obtained by the method just described there has been added (*a*, *b*, — · — · — · —) the result of an attempt to fit the observations by means of the formula proposed by Bĕlehrádek (1926, *a*). This formula, *time* $= A/(t°\text{C.})^B$, (or *rate* $= (t°)^B/A$,) tested by appropriate plotting of the observations as *log rate vs. log* ($t°C.$); in such a plot the best fitting straight line was adjusted, and has then been transferred to the present figure. It is obvious that the fit is anything but significant; nor can the adjustment of the ends of the curve be made such as to improve the fit; from the nature of Bĕlehrádek's formula, correction of the lower end of the curve, for example, merely increases the deviation at the upper end.

Extensive data upon the myogenic heart of *Limulus* larvæ (Crozier and Stier, 1926–27, *b*) have convinced us that the relation of pulsation frequency to temperature is of a perfectly regular sort, with definite and recurrent values of μ.

VII.

This paper is by intention an introductory account of material which it is designed to employ for certain types of experiment. But we may point to several conclusions which the preliminary examinations seem to justify. The rhythmic neuromuscular activities of arthropods, so far as tested, fall into two general categories as regards the critical thermal increments which they reveal. In one class are found movements of heart and of locomotor appendages and the rhythms of stridulation and of luminous display (Crozier, 1924–25, *a;* Crozier and Federighi, 1924–25, *a*, *b*; Crozier and Stier, 1925–26, *a;* Fries, 1926–27; and some other instances as yet unpublished). These we have reason to regard as controlled by nerve centers, and they show a high degree of consistency in providing μ = 12,200 to 12,500. Occasionally this value is associated with a higher one over the lower portion of the temperature range, μ = 18,200 or 23,500. On the other hand, movements of respiration (Crozier and Stier, 1924–25, *a;* and other data unpublished), presumably also determined by nerve center activity, but demonstrably of a different kind, consistently yield other increments,—those, namely, associated with cell respiration itself (Crozier, 1924–25, *b*).

The isolated leg of *Notonecta* with its "heart" is remote from central nervous control of the type pretty certainly involved in the movements of the dorsal vessel (*cf.* Alexandrowicz, 1926). We are not yet able to say just what the thermal relations are in the intact animal, but in the isolated leg-"heart" the increment 12,300 ± does not appear among the several values encountered. On the other hand, the values actually found (Table I) are those characteristic of oxygen utilization, heat production, and CO_2 production. We do not conclude from this, of course, that the leg-"hearts" are therefore concerned with respiration, but merely that the metabolic activities underlying and controlling the rates of pulsation are certainly different from that determining the increment 12,300.

It is notable that in material of this sort we should rather expect serious disturbance of rectilinear relationships between *log rate* and $1/T$, such that plainly curvilinear graphs would be gotten, or progressive shifts of μ. But the fact is that here, as in the case of grasshopper respiratory motions (Crozier and Stier, 1924–25, *a*) and in that of the heart of *Limax* (Crozier and Stier, 1925–26, *b*), when change of μ occurs it takes place abruptly and by a definite amount. This speaks strongly for the individualized character of the several processes which may control the rate of pulsation.

The presence of increments often associated with respiratory phenomena led us to attempt to alter the increment by controlling the oxygen supply. We were unable to obtain consistent differences by comparing hearts in legs immersed in water through which O_2 constantly bubbled, legs swathed in vaseline, or legs sealed in tubes with a small volume of water. The differences found are for the greater part attributable rather to the metabolic condition of the whole insect. This we expected to find influenced by laboratory confinement. It is of interest, therefore, to note that the increments 32,000 ± were chiefly, although by no means exclusively, obtained from individuals not more than 2 weeks after collection. The increments 19,800 and 23,800 appeared only in the cases of legs from *Notonecta* kept for some 2 months or more in the laboratory.

VIII.

SUMMARY.

The frequencies of pulsation of the "accessory hearts" in the isolated swimming-legs of *Notonecta* were studied in relation to temperature, with the idea that in such organs central nervous control is impossible, and that in an isolated system irreversibly proceeding toward death it might be expected that further evidence would be found regarding the supposed specific significance of critical thermal increments. A number of values of μ are found, commonly 8,200; 16,200; or 32,200; less frequently 11,400; 19,800; and 24,500. These values are definitely contrasted with that (12,300) typical for heart beat frequencies in arthropods. They exhibit interrelationships of the sorts already found in other cases. There occur also sharp irreversible changes in frequency of pulsation, which may or may not be accompanied by change of increment. The net result is held to be confirmatory of the interpretation of thermal relations proposed in earlier papers.

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TEMPERATURE AND FREQUENCY OF CARDIAC CONTRACTIONS IN EMBRYOS OF LIMULUS.

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I.

The heart of *Limulus* is classic as example of neurogenic cardiac rhythm (Carlson, 1905-06, 1909). The frequency of its pulsations may be controlled by influences affecting the heart ganglion alone. When the temperature of the ganglion alone is varied (Garrey, 1920–21, *a*, *b*, *c;* 1921–22) the frequency of contractions adheres to the Arrhenius formula, μ in the equation

$$\textit{Frequency} = Ke^{-\frac{\mu}{RT}} + C$$

being = 12,200 (Crozier, 1924–25, *a*); in some preparations there occurs a "break" at 15°, with μ at lower temperatures = approximately 23,500.

In the embryo of *Limulus* there is an early developmental period in which the heart is visible, contracting rhythmically, while the cardiac nervous system is still unformed; during this interval the heart rhythm is "myogenic" (Carlson and Meek, 1908). We have determined the relationship between temperature and frequency of heart beat during this period of "myogenic" rhythm, in order to compare the temperature effect with that in the heart ganglion of the adult. There seem to be clearly defined differences.

There is no reason to suppose that a difference in μ obtained in this way necessarily corresponds to or is diagnostic of myogenic as contrasted with intrinsically neurogenic processes. In so far as values of μ appear to be specific, and thus to correspond to physical realities which may be utilized for purposes of classification and analysis,[1] it must be held that particular magnitudes of μ may reappear in all sorts of situations and do not pertain individually to particular types of function. But at the same time it is apparent that the occurrence of different systems of temperature characteristics for the two cases, embryonic heart and adult, is fully consistent with the idea that the respective essential controlling processes are unlike.

Such a result is of course not unexpected. The metabolic state of embryonic cells must differ materially from that of relatively greater dynamic stability enjoyed by the protoplasm of fully differentiated tissues. From this standpoint the apparently "irregular" variation of μ for rhythmic contraction in cultures of explanted chick myocardium (Murray, 1925–26) might be understood without reference to obscure regulation by the organism as a whole in order to account for greater uniformity in results when organs of intact animals are observed, even without appeal to structural conditions. When whole organisms are used it is possible to obtain modifications of temperature characteristics (μ), and these modifications appear to be specific (Crozier and Stier, 1924–25, *a;* 1925–26, *b*). It is entirely possible that the regularity of μ for comparable activities is partly determined by structural conditions in normal organs, such as permit of active control by definite "pace makers." Certain effects which seem to necessitate this view are discussed in a later section. It is perhaps of interest for this interpretation that the frequency of pulsation in the hearts of intact embryos may show considerable differences in μ among similar individuals (*cf.* Crozier and Hubbs, 1924, and other cases), or in relation to age and other variables. The present observations show differences between individuals comparable to those experimentally induced in the breathing rhythm of the grasshopper (Crozier and Stier, 1924–25, *a*).

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[1] Crozier, 1924–25, *a, b;* 1925–26, *b.* Crozier and Stier, 1924–25, *a;* 1925–26, *a, b.* Fries, 1926–27. It may be noted that change of μ coincident with the institution of neurogenic control would not necessarily prove diagnostic either, for we should require study of comparable developmental stages in the absence of nervous elements.

II.

The heart of *Limulus* embryos within the egg envelopes becomes visible as a pulsating organ at the stage labelled *H* in accounts of the differentiation of the embryo (Kingsley, 1893; Kishinouye,1893). For a period of about 6 days, or until Stage *K* (before the appearance of the telson), at laboratory temperature (Carlson and Meek, 1908), the activity of the heart continues to be visible, in the absence of nervous control.

The heart is not at any time particularly easy to see. This difficulty, together with expected variation in the effect of temperature, led us to practice special precautions in obtaining a large number of observations. The frequency of the heart beat is made visible by horizontal light of fair intensity, under which the cardiac tube appears as a delicate white ghost against the yellowish background of the substance of the embryo. In order to maintain the animal in a position suitable for observation, the egg membrane was punctured and a segment of it folded outward. This segment was fastened by white vaseline to a small glass block. The collapsed membrane holds the embryo in a relatively fixed position. Such preparations live in an apparently normal way for many days and continue to develop.

A number of glass blocks, carrying labelled embryos, are placed in a thin walled crystallizing dish with sea water. Into this projects a microscope with paraffined objective used as an immersion lens. Dish and microscope are securely fastened to an iron frame supported on the rim of a large water thermostat. The vessel containing the embryos is submerged so that its water level is below that of the water in the thermostat. The temperature is read on a thermometer with enclosed stem, calibrated, and graduated to 0.05°.

In all such experiments difficulty is met in maintaining temperatures below that of the room. The latitude of fluctuation in frequency of contractions, at constant temperature, necessitates a number of observations at uniform temperatures on each embryo. Yet it is required to change the temperature by a known small amount at intervals of about an hour.

To do this we constructed a thermostat from which heat could be abstracted by a SO_2 compression circuit. The coil and brine tank of a refrigerating unit were replaced by a considerable length of half-inch Cu tubing, coiled so as to form a helical shell within the wall of a 10 gallon glass tank. The motor operating the compressor was started through a relay actuated by a large mercury thermoregulator.[2] "Sticking" and sparking were obviated by using a Ni-steel needle to make contact with the fluctuating Hg surface, and by having the relay of high resistance (5000 ohms).[3] With adequate stirring, and felt insulation, this device enabled us to maintain for as long as desired any required temperature between 0° and that of the room. When an electric heater, constant or relay-controlled, is added to this arrangement, temperatures above that of the room are similarly obtainable, and the slight lag in temperature adjustment is still further reduced. The constancy of temperature within the body of the thermostat is then ±0.001°. In the vessel carrying the objects to be studied the constancy is well within 0.01°, and this is improved by a cover.

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[2] For aid in this construction we are greatly indebted to Mr. H. V. Rivinius, refrigeration engineer of the Metropolitan Ice Co.

The temperature is changed quickly by adding hot or cold water from a reservoir, excess in the thermostat being removed by a constant level device. The thermoregulator is readjusted by sucking Hg out of the regulator bulb, or by forcing more Hg into it from an accessory bulb. Finer adjustment is made by the screw-mounted contact needle. This operation required but a minute or so.

The thermostat is mounted upon a box, and a window is left in the insulation of the bottom. Through this window a beam of light is projected vertically upward, and may assist observation through the microscope or be employed in other ways.

The microscope, of ordinary type or a binocular with "Planktonsucher" objectives, is so mounted as to be movable over the observation chamber; but in the present experiments it was found that the glass blocks bearing embryos could easily be manipulated with a needle and in turn brought into position for the readings. These movements were found to be without influence upon the frequency of the heart beat, but some minutes were allowed to elapse before readings were taken. Lateral illumination was supplied by a small submerged electric lamp. It was easily shown that the light was without effect upon the frequency of contractions, but the general illumination was kept reduced as an aid to seeing the heart, thermometers, and thermoregulator being viewed by means of small accessory lamps.

III.

When the frequency of the heart beat or of breathing movements is to be timed with precision in an intact animal it is necessary to avoid carefully the effects of concurrent movements of the body or appendages.[4] In some instances it seems as if the execution of such movements is the cause of accelerations or retardations in the rhythm under observation; in others, it appears more probable that both disturbances have a common and simultaneous origin in the central nervous system. If it is desired to study intrinsic fluctuations of frequencies and to obtain temperature characteristics as precisely as possible, such deviations must be taken account of. In the *Limulus* embryo, as in other embryos, there are evident from time to time "spontaneous" movements of body and legs. But it happens that in *Limulus* these movements, like the photokinetic movements of the legs which appear when the light intensity is suddenly changed, seem to have no influence whatever upon the sequence of the heart beats.

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[3] Regulation may also be conveniently made by means of a system such as that described by Beaver and Beaver (1923).

[4] *Cf.* Crozier and Stier, 1924–25, *a*.

The frequency and the amplitude of the cardiac movements nevertheless go through a rather definite cycle, at constant temperature. This was ascertained by repeated observations on single individuals; since the data add nothing new to the theory of the case, they are not given here. The latitude of variation is slightly greater than 10 per cent of the mean frequency, and, as in some similar cases earlier described, is constant over the workable temperature range but varies from one individual to another. The latitude of variation seems quite unrelated, in general, to the relative rate of the process considered, and since it varies within pretty narrow limits for a variety of activities (5 to 10 per cent of the mean), it must be regarded as chiefly determined through some property of protoplasmic organization rather than by the specific process whose temperature characteristic is being measured. In a small number of known cases (Crozier and Stier, 1926–27, *a*) the latitude changes when μ differs on either side of a critical temperature, and in such cases a specific association must be assumed.

The slow developmental pace of the *Limulus* larva, together with the great resistance to asphyxiation (*cf.* Kingsley, 1893; Newman, 1906; Redfield and Hurd, 1925) contribute to its suitability for our purpose. The majority of the individuals used were kept at 20°, when not subjected to temperature changes experimentally. No differences were seen in other embryos maintained at 4.5° for several weeks. It was possible in this way to have embryos of various stages of development available at one time. Reversing the course of the temperature changes gave observations in good agreement. Within certain limits duplicate "runs" on successive days also agreed well; but as a rule the frequency of heart beat changed after 1 or more days, although—so far as ascertained—without change of temperature characteristic. The total number of observations was 3400.

IV.

Sixteen series of readings, on fourteen different individuals, were well controlled by repeated check observations at the same temperatures. Of these individuals seven provide increments ranging from 11,000 to 12,280 (the latter value is probably too high); for these the average μ = 11,520 ± 100. Three series gave μ = 16,430 ± 200.

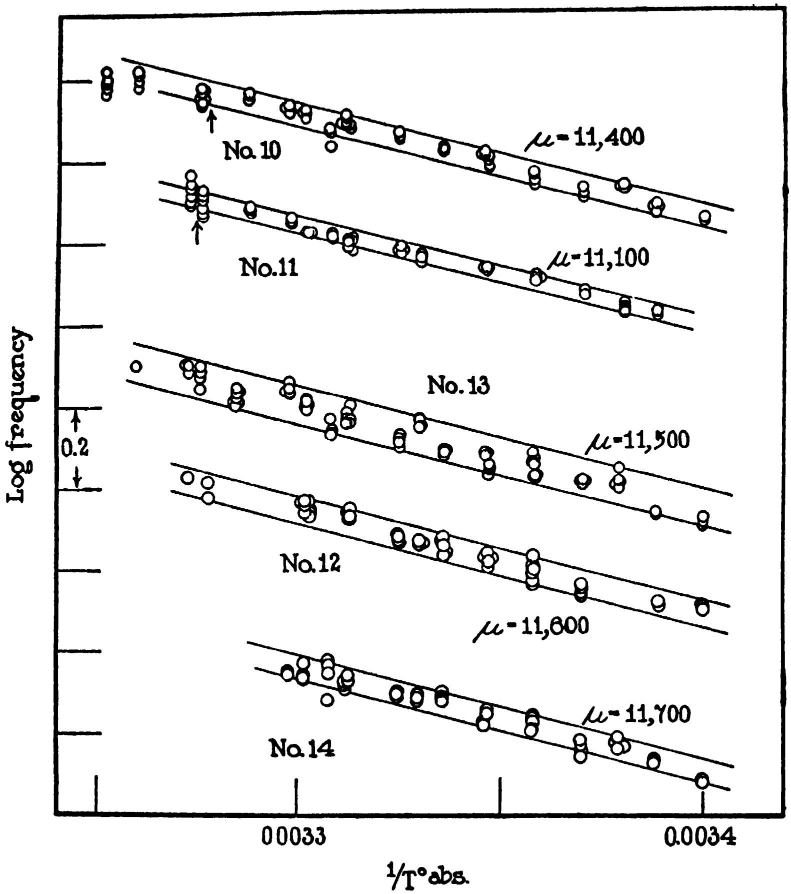

FIG. 1. Observations on the frequency of heart beat in five embryos of *Limulus*, at temperatures between 20° and 35°±, for which μ = 11,400. (The frequencies at constant temperature are very nearly the same; the frequency is taken as 100 ÷ seconds for ten beats.)

Two gave $\mu = 20{,}000 \pm 100$, and two μ 25,500 ± 300. When data from any one animal are considered over a range of temperatures the lower value of μ pertains to the higher temperature interval (20°–30° +), but one embryo gave $\mu = 11{,}000$ over the range 10°–20°, and $\mu = 16{,}400$ occurs both in the range 10°–20° and in 20°–30° (with different embryos).

Above 30°–34° the rate of increasing frequency of heart beat with elevation of temperature is very slight; most embryos of this age show slight but easily detected decreases in frequency of heart contraction, which are only very slowly reversible on return to temperatures below 30°. Other individuals do not exhibit this hysteresis, but above 30°± the thermal increment is very small ($\mu = 5{,}000$ ±) between 30° and 40.5°. This effect resembles that already noted in some other instances (Crozier, 1925–26, *b*), and is suggestive of the control of heart beat frequency by some purely physical condition, such as fluidity of substance or the saturation of some reactive sur-

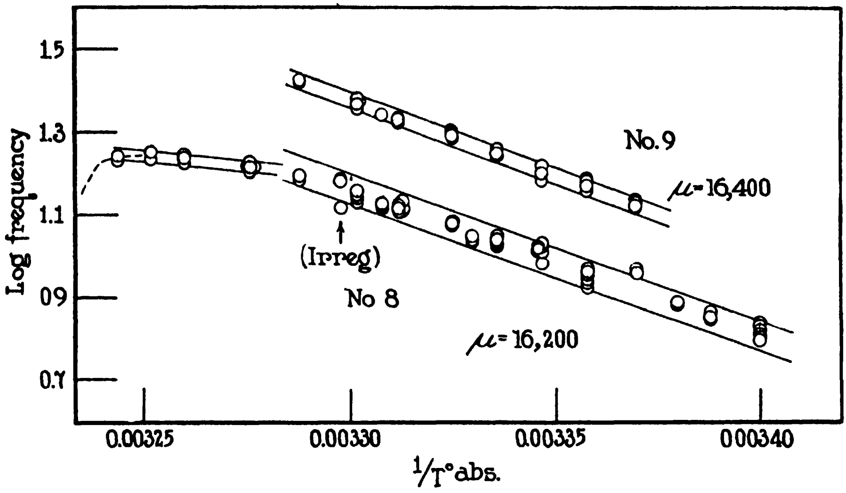

FIG. 2. Data from two *Limulus* embryos for which $\mu = 16{,}300$ (20°± to 30°±); the rates for No. 9 have been multiplied by 1.59. The latitude of variation, as seen also in Fig. 1, varies with the individual. Above 30°± the frequency changes very little with increasing temperature; the curve, within the region shown (No. 8) is perfectly reversible. This phenomenon has already been referred to as apparent in other cases (*cf.* Crozier, 1925–26, *a*).

face which gives a mechanical limit to the maximum frequency of pulsation. In one case which is illustrated (Animal 2, Fig. 5) no hysteresis was apparent on return to lower temperatures.

The temperatures for cessation of regular cardiac rhythm were 9° and 45°; above 40.5° the frequency of contraction decreases; at 44.4° the heart beat was still regular, but at 45.4° only an occasional beat was apparent. For the adult *Limulus* (Carlson, 1906) the thermal limits for contraction of the heart muscle are given as 0°± and 32°, with "heat rigor" appearing at 47°; for the cardiac ganglion and nerves, the limits of activity are 0°– and 42°–43°. At the lower temperatures, beating was observed to become regular at 8.7° to 9.2°; below 8.7°, down to 5.9°, an occasional isolated beat was seen; on warming up to 8.8° to 9.0°, regular contractions were uniformly observed. It is worth noting that these thermal limits, determined from observations upon a large number of embryos, were found to be the same in embryos kept for 15 days at 4.0° as in those maintained at room temperature or used in warming or cooling experi-

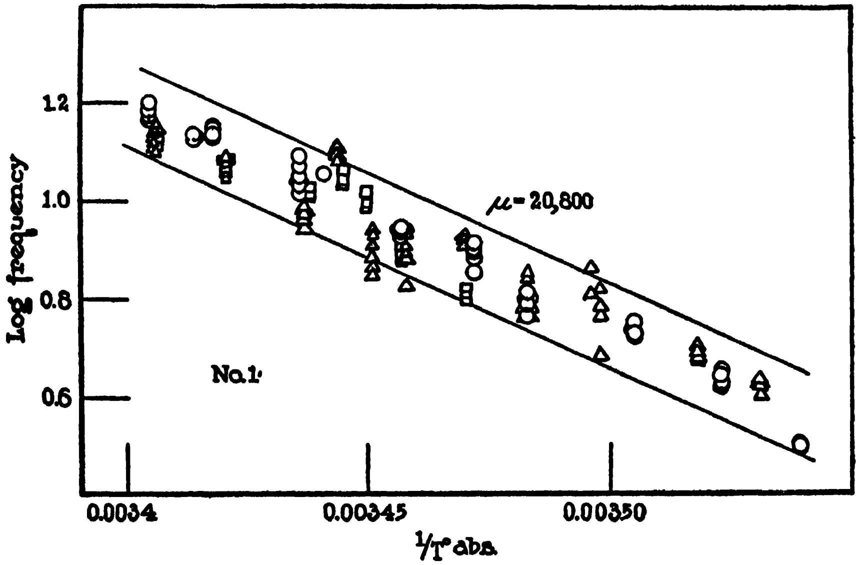

FIG. 3. One individual gave data (three "runs" of observations) yielding μ = 20,800 for frequency of cardiac rhythm, below 20°.

ments. This agrees with the essence of Mayer's (1914) findings upon the adult *Limulus* and points to the conclusion that in this instance the thermal effects depend upon the composition of the animal, rather than upon thermal adaptation.

Between the extremes of temperature which limit the exhibition of regular rhythm (9°, 40.5°) the following are found to be critical temperatures, in the sense (Crozier, 1925–26, *a*) that abrupt change or irregularity may there appear in the curve relating frequency to temperature: 20°, 27°, 30° to 34.5°. We purposely avoided the possible influence of the "break" at 20°± by largely working below or above this temperature, with different embryos. One individual (No. 2; Fig. 5) was found by repeated runs of observations to exhibit a sharp "break" in the curve of heart beat frequency at 20°–21° of such a character that a change in frequency accompanied a change of temperature characteristic. This is the first instance of the kind which we have been able to study carefully. The probable existence of such cases has earlier been mentioned (Crozier, 1925–26, *b*); they are of particular interest for the theory of critical temperatures.

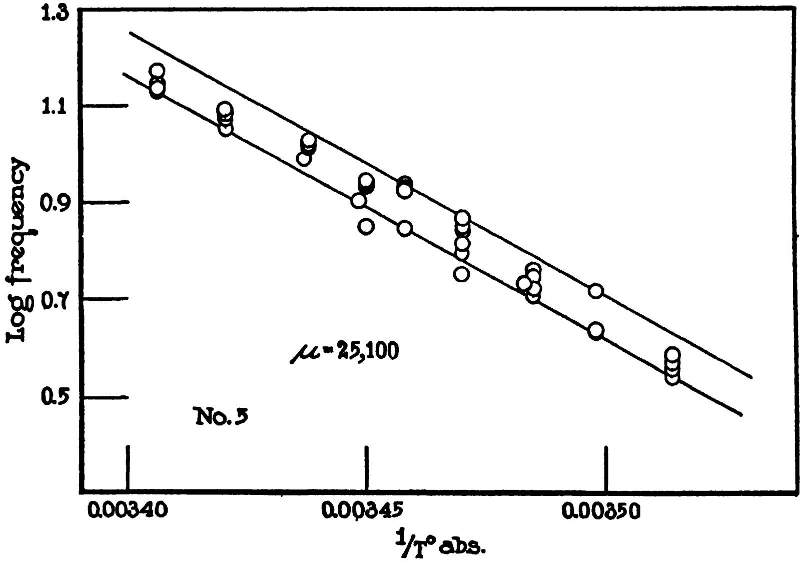

FIG. 4. *Limulus* Embryo 5 gave μ = 25,100, below 20°.

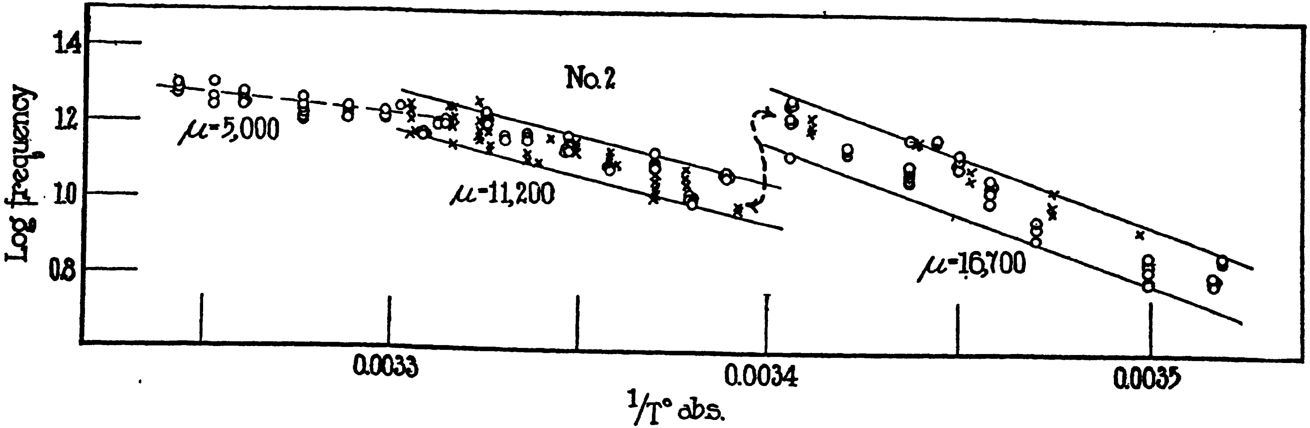

FIG. 5. Embryo 1 gave μ = 16,700 below 20°, μ = 11,200 between 20° and 30°; above 30° the curve flattens out (as with No. 8, Fig. 2, and in other instances not plotted). The particular interest of this graph is in the type of "break" found at 20° (see text). Two series of observations are distinguished by symbols; in the second series (crosses) the latitude of variation is less, below 20°, than in the first series.

V.

The temperature characteristics for frequency of cardiac rhythm in *Limulus* embryos thus appear as definite and recurrent quantities. The fact that two individuals apparently similar may yield quite different magnitudes of μ simply means, we take it, that the two pace maker cells or cell groups in these hearts have slightly different metabolic adjustments, or that the pace maker groups are different. If this were correct we might reasonably expect to alter μ experimentally in a more or less predictable way. This we did not attempt in the present observations although it has been accomplished in other instances (Crozier and Stier, 1925–26, *b*). It would also be predicted that the various values of μ obtained should show certain interrelationships. Thus the common association of the values 11,300 and 16,200 in respiratory and other processes (Crozier, 1924–25, *b*) finds rational application in the present case.

For the heart of adult *Limulus* the characteristic μ, so far as can be ascertained, is about 12,200; in several individuals (data from Garrey, 1920–21, *a*, *b*; *cf.* Crozier, 1924–25, *a*) μ = 23,500 below 15°. With one exception, which is not intrinsically of great weight, this value of μ does not appear in connection with the embryonic heart. The data upon adult heart rates came from experiments in which the temperature of the cardiac ganglion alone was varied, and the μ obtained agrees quantitatively with that for a number of other instances among arthropods in which central nervous control may be assumed (Crozier, 1924–25, *a*; Crozier and Stier, 1925–26, *a;* Fries, 1926–27). The increments apparent in the observations on the embryonic hearts, however, are of frequent occurrence in data on the heart rhythms of molluscs and vertebrates (*cf.* Crozier 1925–26, *b*). This sort of result points definitely to different chemical control of heart pulsation in embryo and in adult *Limulus*, and to the relative diversity of the pace-making control in the developing embryos. (If adult *Limulus* were to be used, the possibility of myogenic effects might have to be reckoned with, in addition to the neurogenic automatism, if the temperature of the whole organism were varied; *cf.* Hoshino, 1925.)

The possibility of diverse pace-making processes in the hearts of different individual embryos, and thus in different but functionally analogous cells, is important for the understanding of thermal effects in isolated heart preparations and in cultures of developing myocardium. To this there must be added the recognition of sources of confusion which may result from the fluctuation of controlling circumstances within single cells, of which at least two kinds of disturbing effects can reasonably be suggested. The behavior of the "accessory hearts" of *Notonecta* is particularly significant in this connection (Crozier and Stier, 1926–27, *b*). At the moment we wish to deal particularly with the consequences of the occurrence in a single pulsating structural mass of a diversity of possible pace-making elements (*cf.*, for the chick heart, Cohn, 1925). Murray (1925–26) found that the apparent temperature characteristics for frequency of pulsation in cultured explants of chick myocardium failed to show uniformity, and failed to be grouped about detectable modal values. In such preparations there must exist at any moment a number of possible pace makers. The net result of their fluctuating control would be expected to obscure or to blur the influence of any one, since it is fair to assume that their respective inner metabolic states might be differently adjusted. In heart cell cultures the controlling influence of the intrinsically faster beating component of compound masses has been demonstrated experimentally by Olivio (1926), and this appears indeed to be a general condition (*cf.* Mayer, 1911; Crozier, 1916).

The sort of situation, therefore, which we believe to exist in pulsating heart cell cultures is one in which a number of distinct "pace-making" cells or cell groups are present in each pulsating mass. The intrinsic frequency of initiation of rhythmic contraction is supposed to differ among these pace makers. If one pace maker definitely possesses a much faster rhythm than the others, its effect is uniformly apparent. But if two or more pace makers have nearly the same intrinsic frequencies, but are metabolically different, so that each exhibits a characteristic relation to temperature, their several influences upon the gross sequence of pulsations should interpenetrate; at one moment pace maker *A*, at another instant, before *A* starts again, pace maker *B* is in control. One consequence of this kind of effect may be tested immediately. The latitude of variation, expressed as a percentage of the mean pulsation-frequency at each temperature, should not be constant if pace makers *A* and *B* have different temperature characteristics. This is precisely the situation disclosed in several of Murray's (1925–26) figures. Therefore temperature characteristics deduced from such data *en mass* must be regarded as without specific significance.

Three corollaries are at once deducible. (1) The situation here pictured and tested differs from that (Crozier, 1924–25, *a*) in which it is supposed that the slowest process of a catenary series of catalyzed transformations dictates the speed and temperature characteristic for the velocity of formation of the end result. In the present case the swiftest pace maker determines the maximum frequency of rhythmic contraction. Therefore we may expect to find cases in which the "mean" temperature characteristic is increased at higher temperatures. The realization of this possibility is suggested in several figures given by Murray (1925–26; Figs. 1, 2, 3). One effect of this would be to bring about instances in which the log latitude of variation increases with increasing temperature as well as others in which the change is in reverse direction. These differences appear in Murray's figures.

(2) Of greater interest is the corollary that if our assumed pace makers *A* and *B*, respectively generating the most frequent and the least frequent contractions among the group capable of acting as pace makers at all, should have the same temperature characteristic, then the logarithmic latitude of variation should be constant; and in such cases the μ deduced should correspond well with a value found in homologous instances. In the figures given by Murray (1925–26; Figs. 1, 2, 3) we find, for cases meeting this requirement, μ = 8,000, μ = 11,000, and μ = 16,100; these compare well with values commonly recognized in heart rate measurements (Crozier, 1925–26, *b*).

(3) For cases in which the latitude of variation is inconstant, the slopes of the lines fitting the extreme variates on the semilog plots should be straight and should provide approximate μ values characteristic of the limiting pace makers. And we should expect these to compare favorably with temperature characteristics encountered elsewhere. We have treated in this way the plots given in Murray's Figs. 1, 2, 3. The result in one case is reproduced in Fig. 6. The

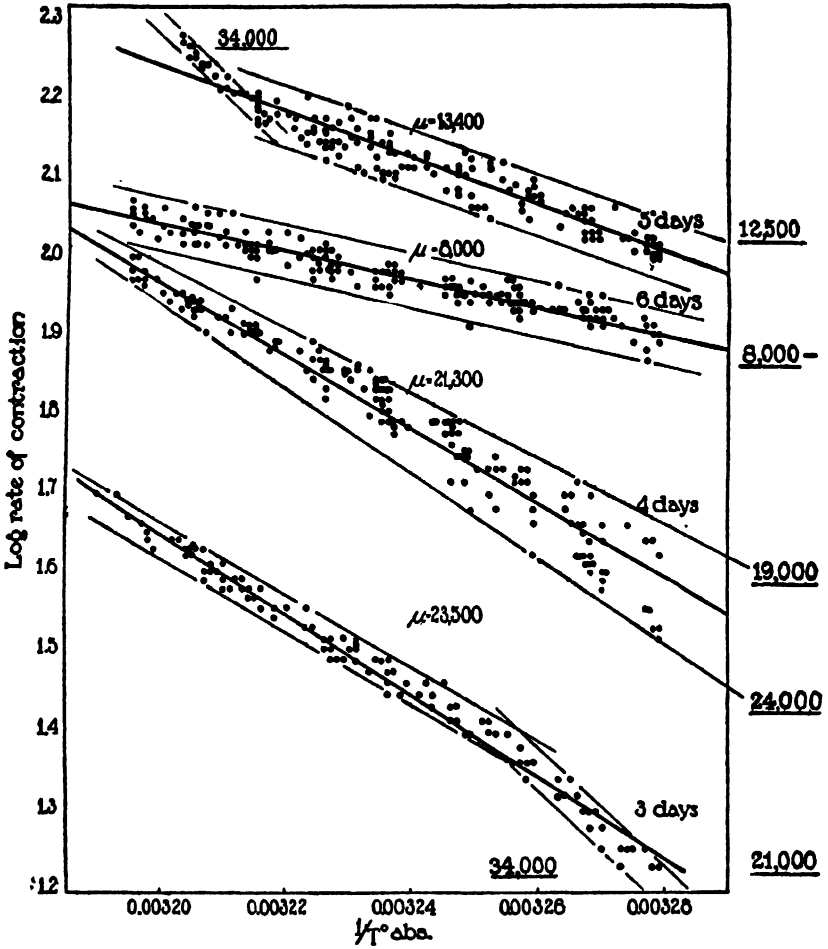

FIG. 6. Reproduced from Murray (1925–26, Fig. 2). The contraction rates of auricular fragments from embryos of the ages indicated. The central line in each plot is that originally given by Murray. To these lines there have been added marginal lines fitting the extreme variates. When attention is given to the latitude of variation it is obviously necessary to deduce values of the temperature characteristics somewhat different from those originally given. Values obtained from the marginal lines are indicated with underscoring. The nature of "breaks" in the uppermost and the lowermost graphs are fairly clear. When the log latitude of variation is constant, values of μ are gotten ("6 days," *e.g.*) which are already well known in other situations. When the latitude of variation changes continuously on the semilog plot the marginal rates are regarded (see text) as due to the operation of diverse pace makers. We consider that the recognition of these sources of confusion in curve fitting is sufficient to remove the force of the contention that temperature characteristics vary at random in this case.

characteristics obtained are noted in the figure, and the result may certainly be taken to agree with the expectation.

The effect of two such limiting pace makers could be imitated by combining the observations from two individuals (*Limulus*) in which μ for frequency of heart beat is different but the rates at given temperature approximately the same. It may be suggested that precisely this condition may appear if one were to measure the frequencies of contraction in the heart of an Ascidian, without reference to the places of origin of the individual beats; or, perhaps better, in a medusa deprived of all but several rhopalia.

These considerations do not completely account for the sources of complexity probably present when such an object as a heart cell culture is studied in this way. The indications already obtained, however, show why in these analyses we have continuously insisted (1) upon the errors which may be involved in the process of averaging rates or frequencies of vital processes in different individuals, or even in the same individual at different times, and (2) upon the ribbon form of significant plottings. There is to be added the further type of difficulty entering when a break occurs in the curve relating frequency or rate to temperature; should this sort of change be present in the activity of one pace maker, absent in others, the logarithmic latitude of variation must again change if the curve for this pace maker falls outside the limits set by the activities of other concurrently effective pace makers. Such a break, furthermore, may or may not be accompanied by an abrupt change of frequency; and changes of frequency may occur without change of temperature characteristic. These are not imaginary situations (*cf.* Crozier, 1925–26, *b;* Crozier and Stier, 1924–25, *b;* 1925–26, *b*). It seems to us inherently probable that disturbances of these types are likely to be encountered with greater frequency in objects such as isolated cell masses in culture than in connection with organs of intact animals, although we may also suggest their probable occurrence in the heart rhythms of embryos. The plottings given by Murray (1925–26) contain features suggestive in this respect, which we venture to predict will find explanation in further studies of embryonic heart rhythm.

There is one general aspect of this whole matter which requires brief additional comment. Murray (1925–26) has suggested that the essential difference between the gross results of his observations and those in cases where intact organisms have been employed lies in the operation of some regulatory property of the complete organism. Since there is a possibility of vagueness in the understanding of such

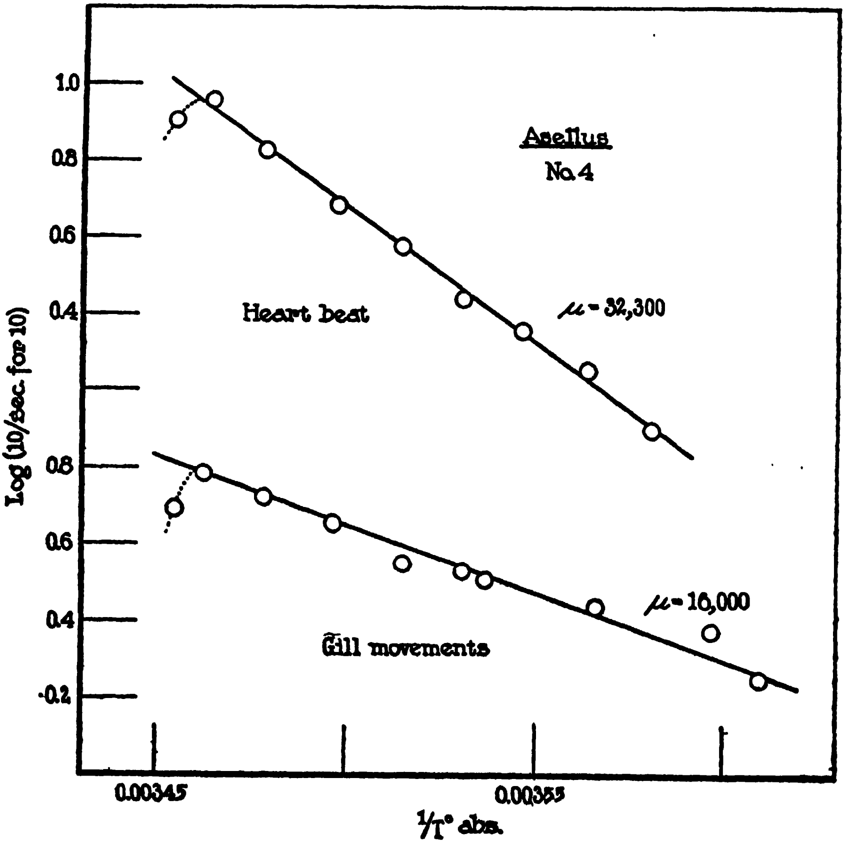

FIG. 7. Simultaneous determinations of frequency of gill movements and of heart beat in *Asellus* show that the temperature characteristic is not the same for the two activities. This disproves determination of μ by the organism as a whole.

a suggestion we may point out that it may be understood in two ways open to test. The "organization factor" might pertain simply to the heart or other structure immediately implicated in the observations; or it might be taken as a feature of the organism as a whole.

The former effect can and must be granted at once as an obvious truism, in the sense that the structure of a heart, for example, permits control by a definite localized pace maker. The latter view can be tested by determining simultaneously the temperature characteristics for two or more different activities in the same individual. We have previously made such experiments. The frequencies of heart beat and of respiratory movements in the same individual arthropod, synchronously determined, do not vary together and do not have the same temperature characteristics. This holds also for embryonic *Limulus*, the frequencies of gill movements providing increments quite different from those here obtained for the hearts (*cf.* Crozier and Stier, in a subsequent paper). For the moment we may illustrate the point by means of data from experiments with *Asellus* (Fig. 7). Therefore a general control by the whole organism is excluded. The results of these experiments will be detailed in another place. They are patently significant for the theory that a specific thermal increment has a particulate locus.

SUMMARY.

Temperature characteristics for frequency of myogenic heart beat in *Limulus* embryos, before the onset of nervous control of the heart, were found to be 11,500; 16,400; 20,000; 25,500. The two first values are the best established. The different values pertain to the hearts of different individuals outwardly similar, and to the hearts of single embryos in different parts of the temperature range. These values differ from that known in connection with the control of the heart beat through the cardiac ganglion. The occurrence of critical temperatures, also, is not the same in all embryos. These facts are employed in a discussion of temperature relations in pulsating explants of chick myocardium.

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GEOTROPIC ORIENTATION OF YOUNG RATS.

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(Accepted for publication, January 12, 1927.)

I.

In describing the geotropic conduct of young rats (*Rattus norvegicus*)[1] we have stressed the fact that to obtain data suitable for quantitative treatment of the gravitational orientation it is necessary to employ individuals of the closest possible similarity. This has meant, in practice, employing litter mates from lines long inbred, under external conditions as nearly uniform as possible. The result has demonstrated that, with these precautions taken, it is possible to formulate precisely the connection between the intensity of excitation and the extent of the geotropic orientation. On a creeping plane inclined at angle α to the horizontal, and in which the effective gravitational component is therefore proportional to sin α, the orientation path is one defined by an angle θ on the plane, such that $\theta = K \log \sin \alpha - C$. The precision of the orientation increases according to the same equation. In addition, $-\cos \theta = K \sin \alpha$. The *speed* of progression bears similar relations to the angle α.

It was proposed to interpret these results as signifying that on an inclined plane the rat orients upward until the difference between the work done by the legs of the two sides is reduced to a certain (constant) fraction of the total. It is possible to entertain this view because the differential postures of the legs encourage it, and because they are extended in the plane of creeping. It is supported by the effects of increasing the mass lifted during creeping, as by attaching weights to the base of the animal's tail. This conception of the geotropic excitation controlling the amount of orientation as a proprioceptive matter is strengthened by further findings in this laboratory regarding the orientation of molluscs. Mr. T. J. B. Stier has also observed an identical type of orientation in newts (*Notopthalmus*), with the additional important fact that when the sign of orientation is reversed, and the animal then becomes *positively* geotropic, the angle θ is the same as in the more usual geonegative orientation.

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[1] Crozier and Pincus, 1926; 1926–27, *a*, *b*; Pincus, 1926–27.

An immediate corollary to these findings in rats of one type (*R. norvegicus*) was the possibility that certain genetic or specific differences might find expression by means of the constants in the equations for geotropic behavior. This deduction we now propose to illustrate. As material for this purpose we chose a strain of the roof rat (*Rattus rattus*). Individuals of the proper age, 13 days, were very kindly placed at our disposal by Dr. H. W. Feldman of the Bussey Institution. We are greatly indebted to Professor W. E. Castle, and to Dr. Feldman, for this and other like assistance.

TABLE I.

The angles of upward orientation (θ) during creeping of young *R. rattus* upon a plane inclined at angle α to the horizontal. The values of θ are each the mean of fifteen determinations, three on each of five rats in one litter.

α	θ	P. E. θ, as per cent θ
		per cent
10°	27.0°	8.74
15°	37.1°	5.78
20°	48.1°	3.68
30°	61 9°	2.34
40°	71.0°	1.88
50°	80.1°	1.72
60°	83.5°	1.06

In this rat the geotropic influence could be expected to be more pronounced. Aside from the matter of its persistence into adult life, the relatively greater lengths of the legs, and the somewhat less body weight, were each expected to play a part in modifying the constants of the equations for geotropism.

II.

Five members of one litter, 13 days after birth, were employed in securing the final series of observations collected in Table I. The experiments were made in a dark room, with temperature 20°–23°, following the technique outlined in previous papers.[1]

Within the limits of the probable errors of the means the measured values of θ adhere quite precisely to the relationship already established[1] for the Norway rat, as Fig. 1 shows, and they thus provide an independent confirmation of it. The precision of orientation decreases linearly as log sin α increases (Fig. 2). The observations at the lowermost magnitude of α are necessarily very variable, because the slight geotropic stimulation does not sufficiently inhibit movements orig-

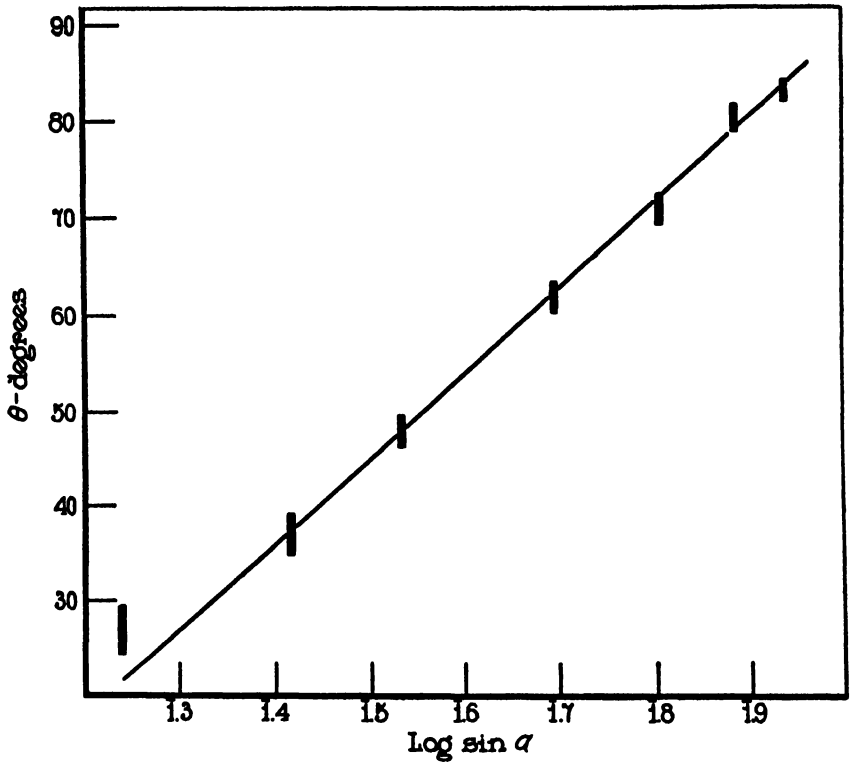

Fig. 1. The extent of upward orientation (θ) on a plane inclined at angle α to the horizontal is linearly related to log sin α during geotropic creeping of young *R. rattus*. Each observed angle of orientation is plotted as a bar of which the height = 2 P.E. The departure of the observed angle at the lowest inclination (10°) is referred to in the text.

inating in other ways. The fact that here again, as with the Norway rat, $\cos\theta$ decreases linearly as $\sin\alpha$ increases, is made evident in Fig. 3.

To compare these results with those given by the species first used it is necessary to obtain the constants in the several equations. This is most conveniently done from Fig. 2. For the roof rat the "ideal threshold" for geotropic orientation is 3.5° [$\cos\theta = 1$], as compared with 6.5° for *norvegicus;* and 90° orientation is obtained at a slightly lower angle (67.2°) than in *norvegicus* (70.0°). This is in accord with the expectation that the "geotropic sensitivity" of the roof rat should be greater. The equation describing the orientation (Fig. 3) is

$$1 - \cos\theta = K\sin\alpha - M \tag{1}$$

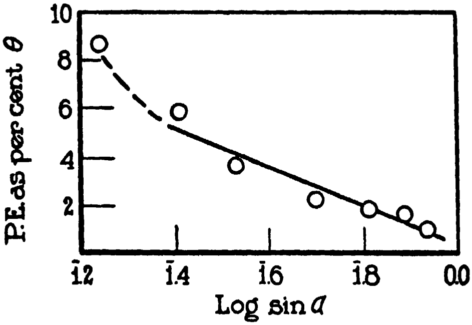

FIG. 2. The variability of the measured angle of orientation (θ) decreases almost linearly in proportion to log sin α. At the lowest inclination the variability is disproportionately large, because the "threshold" presumably differs from moment to moment.

For *R. norvegicus*, of the type used in our previous experiments,[1]

$$K = 1.206$$
$$M = 0.113$$

For *R. rattus*,

$$K = 1.18$$
$$M = 0.06$$

In terms of Fig. 1,

$$\theta = K' \log\sin\alpha - C \tag{2}$$

For *R. norvegicus,*

$$K' = 100$$
$$C = \bar{1}.998$$

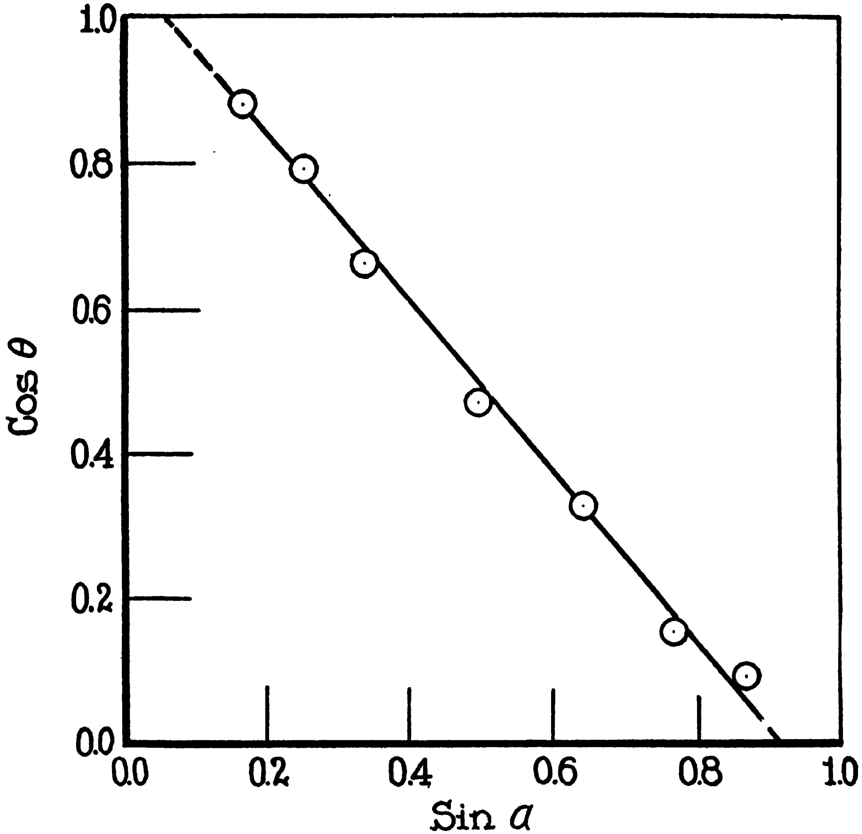

FIG. 3. As with *R. norvegicus* (Crozier and Pincus, 1926–27, *a*, *b*), in *R. rattus* the relationship between cos θ and sin α is rectilinear.

For *R. rattus*

$$K' = 90.4$$
$$C = \bar{2}.988$$

III.

Our sole purpose in recording the outcome of the present experiments is to demonstrate that the methods employed are not only capable of yielding statistically significant results in terms of an intelligible mechanism, but also of providing a method for the precise characterization of behavior differences within genetically uniform strains. It is not too much to hope that the obvious development and application of this view-point may lead to results significant for inheritance studies. Certain aspects of this matter we expect shortly to have in hand.

IV.

SUMMARY.

The geotropic orientation of *Rattus rattus* (roof rat) obeys the equations previously found applicable for *Rattus norvegicus*. The former is more sensitive, geotropically, and the numerical values of the constants in the equations for the two forms are found to differ significantly. Certain consequences of this difference are pointed out.

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GEOTROPIC CREEPING OF YOUNG RATS.

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(Accepted for publication, December 2, 1926.)

I.

The geotropic conduct of young rats has been discussed in a previous paper (Crozier and Pincus, 1926–27) with special reference to the angle of orientation upon an inclined plane. It was found that the angle of orientation (θ) is directly proportional to the logarithm of the gravitational component ($g \sin \alpha$) in the creeping plane. This is explicable as the result of the distribution of the pull of the animal's weight upon the legs of the two sides of the body during progression, upward orientation being the result of the "pull" of the legs on one side and the upward "push" of the legs on the other side; when orientation is attained, the ratio of the tensions on the legs of the opposite sides is regarded as constant and the difference between these tensions as a constant fraction of the total downward pull.

To examine further the nature of the geotropic conduct of young rats, observations on the speed of upward creeping were undertaken. Cole (1925–27) has discussed similar observations on *Helix;* he concludes that the speed of movement, after orientation has been attained, varies as $\sin \alpha$. But, as has been pointed out already (Crozier and Pincus, 1926–27), in these experiments the speed measured was that of *vertical* ascension, and no correction was made for the changes of θ at the different angles of inclination; such changes occur in the orientation of gasteropods. Since, at lower angles of inclination of a creeping plane (15°–70°) the animal moves at an angle (θ), it is necessary to multiply the time of upward creeping by the sine of the angle of orientation (θ) in order that the amount of time actually necessary to cover a constant distance may be dealt with at each angle of inclination (*cf.* Fig. 1). In terms of Fig. 1, the rate of creeping is given by the fraction $\frac{AB}{t \sin \theta}$, where $AB = 32$ cm., and t is time in seconds. The required rates are therefore proportional to $\frac{1}{t \sin \theta}$.

II.

The rats used in these experiments were 13 to 14 days of age. As in the previous experiments, only animals with unopened eyes were used. To insure uniformity the animals employed were of the same age (litter mates), of known genetic constitution, and of the same weight. It was soon found that the animals show periods of activity followed by periods of inactivity, the latter being possibly due to or influenced by fatigue. Care was therefore taken to use only active individuals, and between tests each animal was allowed to rest for 20 to 30 minutes.

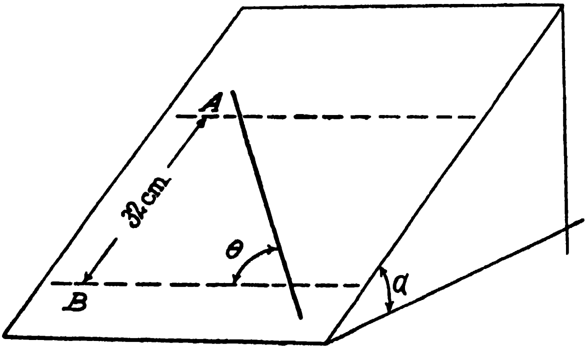

FIG. 1. On a plane inclined at angle α the path of geotropic orientation is (solid line) at angle θ; the time was measured for creeping the distance 32 cm. as indicated. The rate of progression is then obtained as $(AB)/(t \sin \theta)$.

Observations were made in a dark room under red light of low intensity, at a temperature of 23°–25°C. The animals were placed on a creeping plane of wood covered by fine meshed copper wire. A distance of 32 cm. was marked off on the creeping plane with white chalk, and by means of a stop-watch the time was taken for the animal to creep from one white line to another.

At least ten runs were made at each angle of inclination, and at 15° and 20° inclinations, where the speed is more variable, twenty runs were recorded.

III.

Table I contains: (*a*) the times for creeping between two lines 32 cm. apart (*AB* in Fig. 1) as obtained for various angles of inclination from 15°–60°, and (*b*) the corrected rates secured by multiplying the observed rates by the sine of the angle of orientation (θ) and taking the reciprocals. No records for inclination above 60° were taken because these young animals have not the muscular equipment necessary for uniform response to the more intense geotropic excitation of the higher angles of inclination. The speed of creeping, like the angle of orientation, varies directly as the logarithm of the angle of inclination (Fig. 2). Therefore the speed of creeping should be directly proportional to the angle of orientation (θ), and Fig. 3 shows that this is the case.

TABLE I.

Angles of Orientation and Rates of Creeping at Different Inclinations of the Creeping Plane.

Angle of inclination (α)	Time to creep 32 cm. vertically	Probable error as per cent of the mean	Angle of orientation (θ)	Rates of creeping, $\frac{10}{t \sin \theta}$
15°	6.73	3.14	37.4	2.442
20°	5.42	2.94	44.5	2.636
25°	4.73	2.82	52.9	2.654
30°	4.14	2.23	57.4	2.872
35°	3.61	2.42	64.0	3.082
40°	3.40	1.84	69.8	3.139
50°	3.10	1.63	77.9	3.300
60°	2.95	1.05	84.7	3.407

In a preceding paper (Crozier and Pincus, 1926–27) it has been shown that the *precision* of upwardly directed movement increases as the angle of inclination increases. That is, the reduction of variability (V) in the measurements of θ is proportional to the logarithm of the gravitational stimulus:

$$-V = K \log \sin \alpha.$$

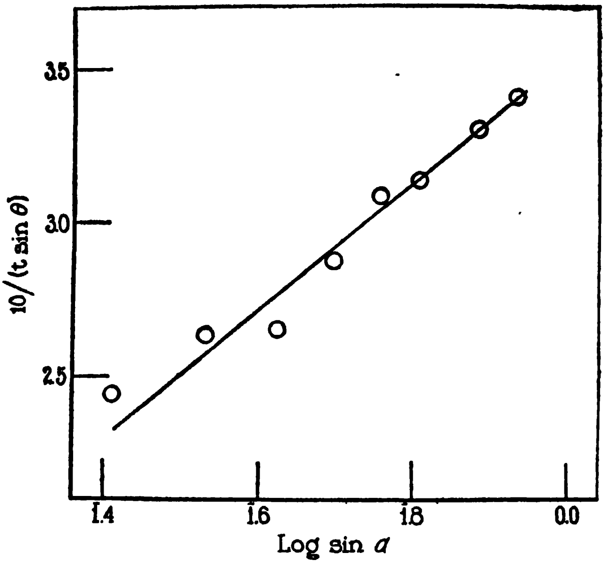

FIG. 2. The rate of creeping expressed as $10/(t \times \sin \theta)$ is plotted against the logarithm of the active gravity component. A direct proportionality is observed, the spread of the points at the lower inclinations indicating the increase in variation with lower intensities of stimulation.

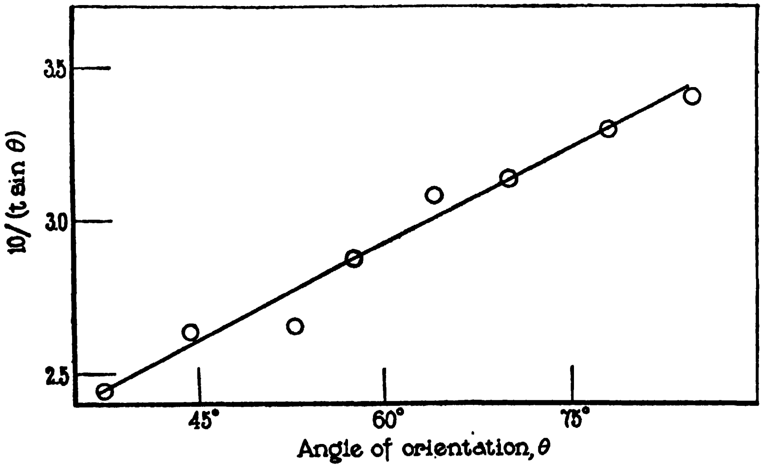

FIG. 3. The rate of creeping is plotted against the angle of orientation (θ). Since both are directly proportional to the logarithm of the active gravity component it follows that they should be directly proportional. This is the case, and a check is had upon the relation expressed in Fig. 2.

This relation is plotted in Fig. 4 for the speed of creeping. The speed of creeping is a much more complicated thing than the simple geotropic orientation, and is influenced by unanalysed fatigue effects and by cycles of activity. Furthermore, only ten observations were taken at each inclination. Nevertheless, it is apparent that the variability in speed of creeping is less at higher angles of inclination than at the lower angles, and that the relationship is linear, as in the case of the amount of upward orientation.

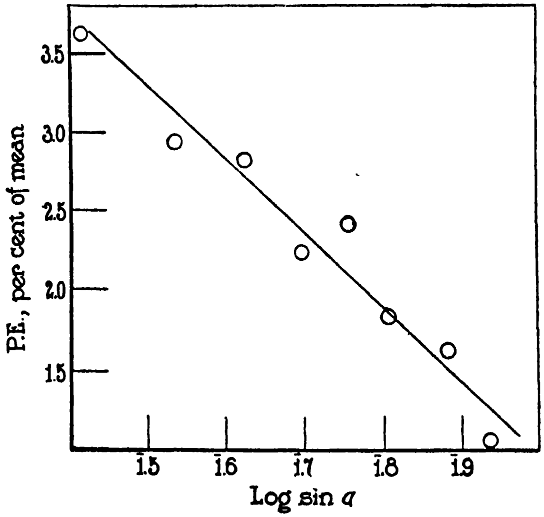

FIG. 4. The "coefficient of variation," expressed by the probable error as per cent of the mean, is plotted against the logarithm of the active gravity component. The result indicates that the variability in the rate of creeping decreases as the angle of inclination (α) is increased, and in the same manner as the variability of the extent of upward orientation (Crozier and Pincus, 1926–27).

IV.

When weights are attached at the base of the tail of a young rat creeping on an inclined plane the angle of orientation increases approximately as the logarithm of the added weight (Crozier and Pincus, 1926–27). To test this relation further, the speed of creeping with attached weights was measured at two angles of inclination, 15° and 20°. Weights of 1.6, 2.6, 5.2, 7.0, and 9.8 gm. were used.

The angle of orientation (θ) with attached weights was measured as well as the rate of creeping, and correction of the rate was made by multiplying the observed times by sin θ, as before. The results are given in Table II. Fig. 5 gives the corrected rate plotted against the logarithm of the added weight and indicates a direct proportionality for the 20° inclination; at the 15° inclination the plot is apparently curvilinear. However, when, as in Fig. 6, the corrected rates are plotted against the angles of orientation (θ) the direct proportionality observed indicates that the curvilinear distribution in Fig. 5 for 15° is accidental.

The significance of these data lies in the fact that they demonstrate the proportionality of geotropic response to the logarithm of the active gravitational component, rather than to the gravitational intensity directly. They give further confirmation of the hypothesis that the geotropic responses are the result of the pull of the animal's weight on the legs of opposite sides. This makes it unnecessary, or indeed impossible, to account for the orientations in terms of the pull of the head upon the neck muscles. It may be emphasized that as the weight of the attached load is increased, or as the angle of inclination of the creeping plane is increased, the legs are actually further extended. The angle of orientation, however, seems to be determined by the difference in effective pull on the legs of the two sides, such that, diagrammatically, at orientation $(x - y) \cos \theta = KMg \sin \alpha$, where x and y represent the lever radii of the legs on the "down" and "up" sides

TABLE II.

Angle of inclination...	15°						20°					
Attached weight (gm.).....	0	1.6	2.6	5.2	7.0	9.7	0	1.6	2.6	5.2	7.0	9.7
Angle of orientation (θ)....	37.35	44.0	51.3	67.0	72.5	84.3	47.95	51.0	62.0	78.1	80.3	90.0
Time of creeping (seconds)..	6.63	5.27	4.50	3.54	3.15	2.77	5.07	4.57	3.64	3.12	2.85	2.65
$10/t \sin \theta$....	2.477	2.734	2.849	3.071	3.332	3.628	2.658	2.816	3.115	3.276	3.562	3.773

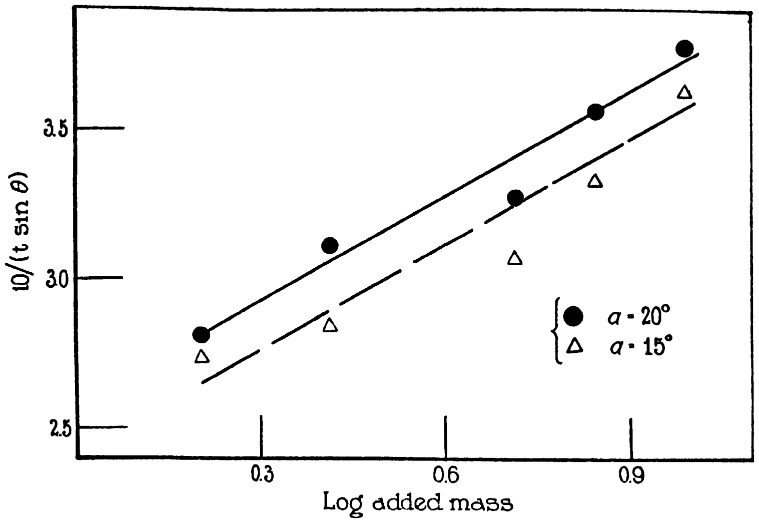

FIG. 5. The rate of creeping with attached weights at two angles of inclination (15° and 20°) is plotted against the logarithm of the attached weight. At 20° the result indicates a direct proportionality. At 15° the result is apparently curvilinear, but this may be due to fortuitous variations in θ.

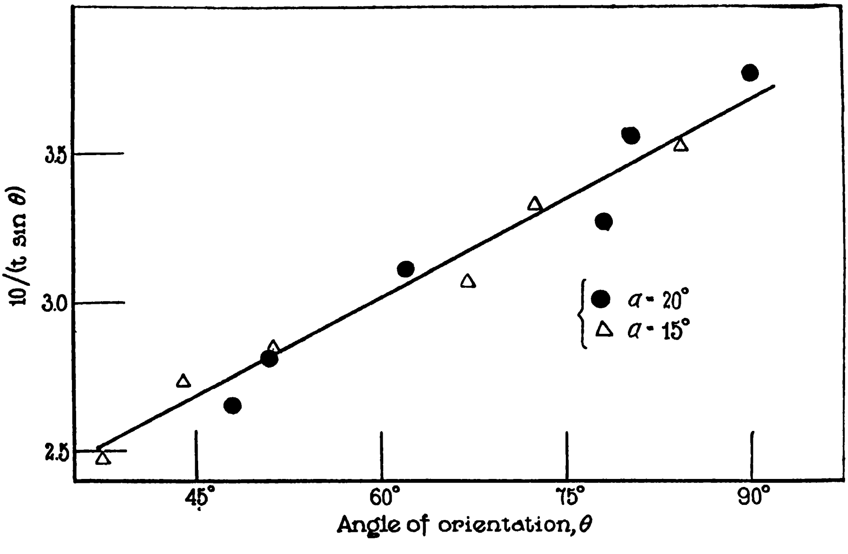

FIG. 6. The rate of creeping with attached weights at two angles of inclination (15°and 20°) is plotted against the observed angles of orientation at these angles of inclination. The result shows a direct proportionality; the curvilinear relation at 15°, as apparently indicated in Fig. 5, is not detectable.

of the body, respectively. If $KMg \sin \alpha$ be increased by adding a load to the rat's tail, K remaining a constant and M being the mass lifted, $(x - y) \cos \theta$ must increase, hence $(x - y)$ must become larger (since θ is increased); therefore the legs on the "up" side are further extended, relatively, than in the absence of added weight; this is a fact of observation. Thus the increase in θ, with α constant, when a weight is added, results from the extension of the limbs by the added load, since the "upper" or y limb is stretched and thus more extended. The speed of creeping in influenced in exactly the same way as the extent of orientation.

SUMMARY.

The rate of upward creeping in negatively geotropic rats aged 13 to 14 days is a function of the gravitational stimulus. The rate of upward movement on the creeping plane, like the angle of orientation, is directly proportional to the logarithm of the gravity component. The variability in the speed of creeping decreases in proportion to the logarithm of the gravitational effect. When weights are attached to the animals' tails the rate of upward creeping varies almost directly as the logarithm of the attached weight, and the speed of creeping is still proportional to the angle of upward orientation.

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POTENTIOMETRIC STUDIES ON INTRACELLULAR pH VALUES OF SINGLE FUNDULUS EGG CELLS.

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Studies on internal reactions of cells as well as permeability of cells to acids and alkalies have usually been concerned with changes in the color of intravital indicators, introduced or taken up by the cell, as well as changes in naturally occurring cellular pigments (1–6). Death and functional changes within cells have also been used as criteria of penetration of acids and other compounds into certain specialized types of cells (2, 7). From the standpoint of an accurate quantitative treatment of intracellular pH changes in such experiments the usual errors and limitations of colorimetric as well as of mortality methods are ever present and seem to allow but limited experimental procedures. The present paper is based upon an attempt to study the intracellular reaction (pH) in single egg cells of a marine fish, *Fundulus heteroclitus*, by means of a micro hydrogen electrode and vessel originally designed for the determination of the pH values of minute quantities of insect blood (8). With suitable modifications of the electrode vessel it has been found possible to make three separate determinations on a single *Fundulus* egg cell.

Method.

The micro hydrogen electrode and vessel used in these experiments were as originally described (8) with the exception of a capillary vessel modified so that a drop as small as 0.01 cc. could be conveniently handled. Both fertilized and unfertilized eggs of *Fundulus heteroclitus*, obtained at Woods Hole, Massachusetts, were used. All eggs were taken directly from the female by "stripping" them into the solution contained in finger bowls. Eggs from each animal were kept and tested separately. In experiments where rates of penetration of acid into the cells were followed the eggs were first thoroughly washed in distilled water to free them as far as possible of adhering electrolytes, as suggested by Loeb (9). In carrying out pH determinations individual eggs were always used and readings were made in triplicate. Between each reading the electrode was washed and checked by readings on standard buffer solutions of known pH values. The electrode vessel was at times put directly into the egg and the fluid drawn up into the capillary, while at other times small punctures into the surface of the egg with fine glass or steel needles were made and the fluid then immediately drawn up into the capillary. No significant differences were found in pH values for fluid drawn up into the capillary by these different methods. In all experiments the time consumed in drawing up the fluid into the capillary and in the taking of pH readings was extremely short, a matter of a few seconds. Eggs were individually taken from solutions by means of pipettes, washed in distilled water in the case of the acid experiments, quickly dried, and excess solution removed from the exterior by placing them on dry filter paper. They were then placed on a small watch-glass, quickly punctured, and pH determinations made on intracellular fluid. In all instances the intracellular fluid alone was used. Twenty to thirty eggs were always used in each experiment with 100 cc. of solution kept in covered finger bowls. Temperature during the entire course of the experiments ranged from 20°–22°C., but for any one experiment did not vary at any time more than ±0.5°C.

Observations and Results.

Results of typical experiments are shown graphically in Figs. 1 to 7. Unfertilized eggs "stripped" from the female directly into distilled water or sea water give the same internal pH values, showing, as repeatedly pointed out by Loeb (9), that the eggs are little affected internally by distilled water. pH values for eggs from the same individual are fairly constant, while eggs from different individuals exhibit considerable variation—as shown in Fig. 1. These variations in eggs from different individuals are doubtless due to differences in the ages of the eggs obtained at the time of "stripping." The mean average internal pH value for unfertilized eggs at the time of "stripping" is 6.39. Unfertilized eggs kept in distilled water or in sea water and the solutions changed at frequent intervals show fairly constant internal pH values for periods up to approximately 48 hours, after which the values become progressively more acid until the egg finally dies. The most striking difference shown between a fertilized and an unfertilized egg is the marked constancy of internal pH values of the fertilized eggs as compared with the variations in the case of the unfertilized eggs (Figs. 1 and 2). This perhaps is again due to the similarity in condition and age of the fertilized as compared with the various degrees of unripeness, etc., of the unfertilized eggs. Fertilized eggs show an extremely constant pH value, 6.39, even after the developing embryo has increased considerably in size (Fig. 2).

It is of some interest to note that the internal reaction of *Fundulus* egg, a vertebrate egg, is acid in nature despite the fact of its almost constant alkaline sea water environment (pH 8.2). Internal acid reactions, however, have been reported for various forms, protozoa, Arbacia eggs, etc. by several authors (1, 3–6).

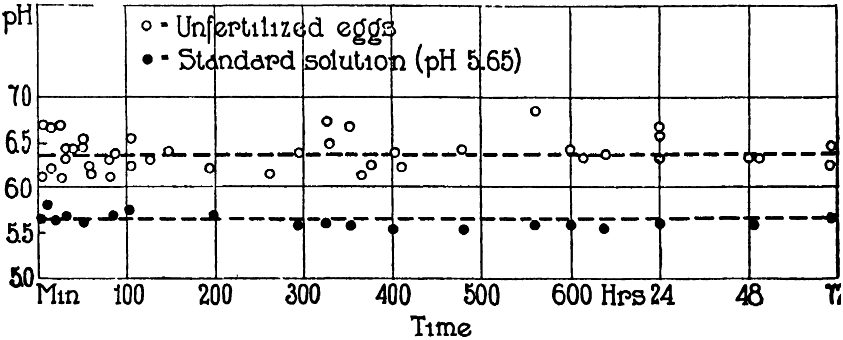

FIG. 1. Shows internal pH values for unfertilized *Fundulus* eggs at time of "stripping" from female and during exposure to sea water for different time intervals. Each point represents average of day, in some cases for eggs from same female, in others for eggs from different females.

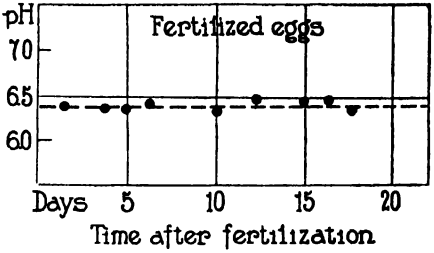

FIG. 2. Shows average internal pH values for fertilized *Fundulus* eggs during course of development of embryo. Points represent average values taken from many individual eggs.

Inasmuch as Loeb (7, 9) has so strikingly shown the extreme resistance of *Fundulus* eggs to changes in osmotic pressure, to acids,

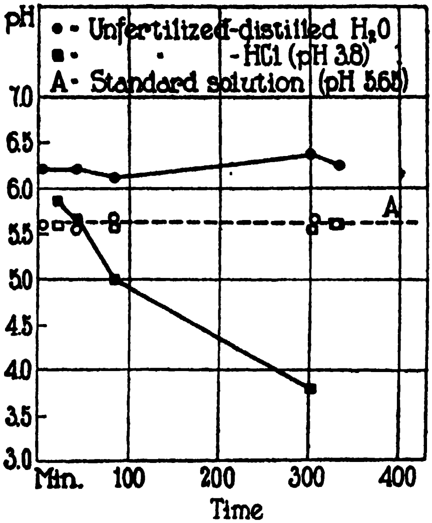

FIG. 3. Shows changes in internal pH values of freshly "stripped" unfertilized *Fundulus* eggs exposed to HCl, pH 3.8.

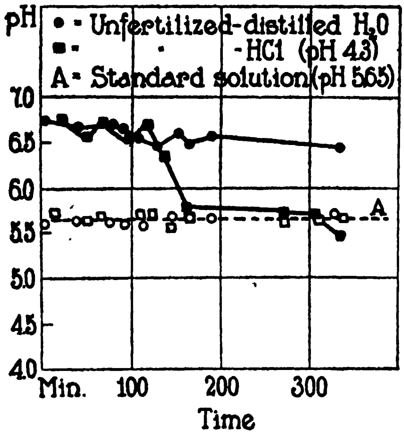

FIG. 4. Shows changes in internal pH values of freshly "stripped" unfertilized *Fundulus* eggs exposed to HCl, pH 4.3.

etc., and since by the present method the internal pH of the egg could be measured, it was thought desirable to make experiments similar to those of Loeb and to follow internal pH changes as well as functional changes in the embryo. Both fertilized and unfertilized eggs were subjected to HCl of different pH values and the rates of internal

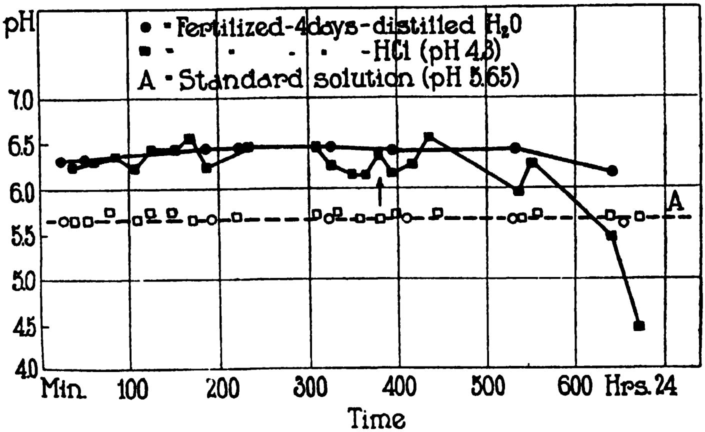

FIG. 5. Shows changes in internal pH values of *Fundulus* eggs (4 days after fertilization) exposed to HCl, pH 4.3. Arrow indicates time of cessation of heart beat and circulation.

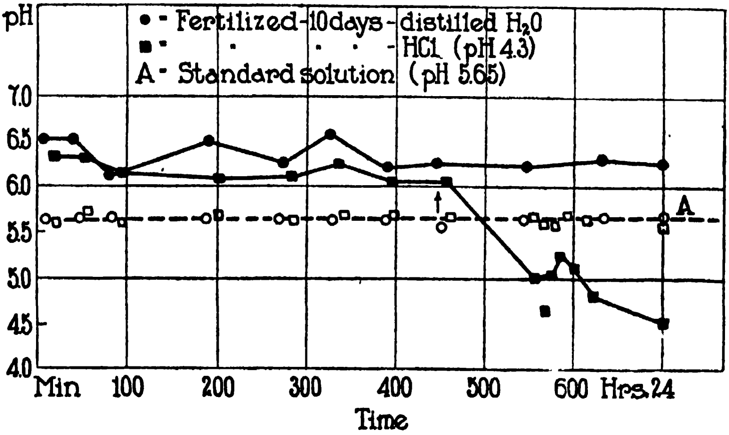

FIG. 6. Same as Fig. 5 but for eggs 10 days after fertilization. Arrow as in Fig. 5.

pH change were noted. Unfertilized eggs seem to be less resistant to HCl than fertilized eggs, as shown in Figs. 5 to 7. It is of interest to note (Figs. 3 and 4) that HCl, pH 3.8, enters the unfertilized egg, as judged by internal pH changes, at a rapid and fairly uniform rate. Changes in opacity of the cells closely follow internal pH changes. Less concentrated acid (pH 4.3) penetrates less quickly, as shown in Fig. 4. No appreciable changes in internal pH occur for some time after exposure to the acid, as shown by the flatness of the curve (Fig. 4) for the first 100 minutes. The fact that the curves show a period during which little penetration of HCl into the egg occurred, followed by a fairly rapid penetration, would seem to indicate a probable surface injury. Fertilized eggs seem even more resistant to HCl (pH 4.3) than unfertilized eggs, as shown in Figs. 3 to 7. No marked differences in resistance correlated with age of the fertilized eggs seem to exist. When changes in internal pH values begin they usually continue at a uniform and slow rate until the external and internal pH values are in equilibrium (Figs. 3 to 7). In all cases at the time when the inner and outer pH values coincided the cell or embryo was dead. Loeb's (7) curves for the rate of penetration of HCl, pH 3.7, into the *Fundulus* egg show during the first few hours of exposure rather a uniform and gradual change in the pH of the external solutions, indicating, according to Loeb, a gradual entrance of the acid into the egg. It seems to the writer that under the conditions

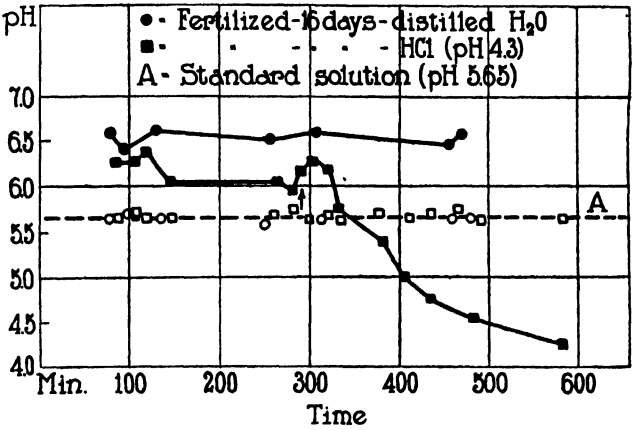

FIG. 7. Same as Fig. 5 but for eggs 16 days after fertilization. Arrow as in Fig. 5.

of Loeb's experiments one would hardly be justified in drawing such conclusions.

In studies on the resistance of *Fundulus* eggs to acid, considerable emphasis has been given by Loeb to the assumption that cessation of heart beat or circulation in the developing embryo indicates entrance of the acid through the egg membrane. During the course of the present experiments all eggs were carefully examined under the microscope and the time of cessation of heart beat or circulation in the embryo noted. It will be found by inspection of Figs. 5 to 7, on which cessation of heart beat is indicated by an arrow, that considerable variations exist as to a correlation between cessation of heart beat and internal pH changes. As a matter of fact, in several experiments it was found that many embryos with hearts stopped could be made to recover by being returned to sea water. It seems to the author quite possible that surface effects of the HCl might easily account for such functional disturbances without internal changes resulting in the pH of the egg. It is, of course, conceivable that the delicate embryonic heart located so close to the surface of the egg might be affected by the HCl before it diffused further into the egg. Such a conception cannot be checked by the present method since we are dealing primarily with changes in the pH of the egg contents and not with the individual cells of the embryo.

These results are of a preliminary nature and show that by this method we may obtain some idea of the rates of entrance of acids like HCl into the egg of *Fundulus*.

SUMMARY.

1. By means of a micro hydrogen electrode and vessel the internal pH values of single egg cells of *Fundulus heteroclitus* have been measured.
2. Unfertilized eggs show a mean average internal pH value of 6.39. Considerable variations in pH values for unfertilized eggs exist and these are perhaps due to variations in the ages of the eggs obtained from different females.
3. Fertilized eggs show a mean average internal pH value of 6.39 with extremely small variations between eggs from different females.

4. No marked differences in pH values for fertilized eggs of different ages were detected.

5. Rates of entrance of HCl, as judged by internal pH changes, have been followed for fertilized and unfertilized eggs.

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4. Vlès, F., Reiss, P., and Vellinger, E., 1924, *Arch. phys. biol.*, iv, 21.
5. Reiss, P., 1924, *Arch. phys. biol.*, iv, 35.
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THE PRECISE MEASUREMENT OF HEMOLYSIN.

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The content in hemolysin of a solution is commonly measured as the amount which is just sufficient to produce complete hemolysis of an arbitrary quantity of red blood cells, usually 0.5 cc. of a 5 per cent suspension of washed erythrocytes. The method employed for such measurement gives results which are neither exactly comparable in determinations made at different times, nor highly precise. The susceptibility of erythrocytes to hemolysis is influenced by many factors, and the amount of the minimal hemolyzing quantity must vary accordingly for every specimen of test cells. Lack of precision in the measurement results from the fact that relative differences in hemolysin content between adjacent tubes in a titration series must be great, in order to distinguish the end-point, so that the value determined differs often by a large amount from a possible true value.

The method of titration of hemolysin described in this paper was developed for a study of the association of hemolysin with different fractions of immune serum and plasma protein. It measures hemolysin content as the ratio of the hemolytic activity of a given solution to that of standard immune serum. This standard has been usually a portion of the whole serum from which isolated protein fractions have been derived.

This choice of a standard immune serum instead of a given quantity of erythrocytes, as the unit of measurement of hemolysin content, has the advantage that applies to the use of a diphtheria antitoxin for the standardization of toxin and of new antitoxin: the antibody is the most stable biological element of the immune system.

The necessity for a large increment of hemolytic substance in successive tubes in a titration to determine the minimal hemolyzing quantity has long been recognized, and is not peculiar to the immune hemolytic system. With most, if not all hemolytic agents the increment of substance necessary to produce the final 10 or 15 per cent of complete hemolysis of a given quantity of cells is not in proportion to that which brings about the preceding fractional amount of hemolysis, but greatly exceeds its proportion. Madsen[1] made this observation first for tetanolysin, and to measure the lytic value of this hemotoxin determined the amount required to produce hemolysis equivalent to that of one-third or one-sixth of the total quantity of red cells used as reagent. Schur[2] used a similar method for the estimation of staphylolysin, and plotted the amounts of lysin against the corresponding amounts of hemoglobin liberated. The S-shaped curve so obtained is given also by serum, saponin, and NaOH, according to Handovsky,[3] although Mioni[4] had previously reported that the amount of serum hemolysis, with an excess of alexin, is proportional to the amount of sensitizer. Brooks[5] found that the amount of hemolysis is not proportional to the amount of alexin in the presence of a constant amount of sensitizer, but is represented by an S-curve which is similar to those of Handovsky,[3] and has devised a method for the titration of complement which makes it possible to compare the amounts of alexin which produce like results in constant time.

The method of titration of hemolysin content described here utilizes the same principle; it compares the amounts of unknown and of standard hemolysin which produce a definite fractional amount of hemolysis of a given specimen of erythrocytes when acting in conjunction with a given specimen of alexin.

The standard immune serum is freshly diluted for each titration and brought to a concentration such that 1.0 cc. will produce almost complete hemolysis of 0.5 cc. of a freshly prepared 5 per cent suspension of sheep erythrocytes. A series of tubes is prepared containing 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 cc. of the standard immune serum dilution. A preliminary titration of the unknown serum or fraction is carried out, if its approximate value is not known, and in a second series of three to five tubes are placed graded amounts of the unknown such that approximately 50 per cent hemolysis of the test cell quantity will be brought about by one of the intermediate tubes in the series. The fluid in all tubes of both series is brought to the same volume, 0.5 cc. of the erythrocyte suspension added with a quantitative or standardized pipette, and alexin representing two units added. For reading in the ordinary Duboscq colorimeter the final volume should be at least 4 cc. Both series of tubes are placed simultaneously into the water bath at 37°C., kept agitated, and withdrawn and placed in cold water as soon as hemolysis is complete or almost complete in the highest concentration of the standard series. After cooling the tubes are centrifugalized and the percentage amount of hemolysis in each tube of standard and unknown series is determined in the colorimeter, using the "highest" tube of the standard series as the 100 per cent standard. This is permissable since complete hemolysis of one "unit" of cells is not the end-point chosen in the comparative measurement.

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[1] Madsen, Th , *Z. Hyg. u. Infectionskrankh.*, 1899, xxxii, 214.

[2] Schur, H., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 89.

[3] Handovsky, H , *Arch. exp. Path. u. Pharmakol.*, 1912, lxix, 412.

[4] Mioni, G., *Ann. Inst. Pasteur*, 1905, xix, 84.

[5] Brooks, S. C., *J. Med. Research*, 1920, xli, 399.

A graph is prepared in which the fractional amount of hemolysis in each tube of the standard series is plotted as ordinate against the corresponding amount of diluted immune serum as abscissa. The curve is S-shaped; it varies slightly in form with each specimen of erythrocytes or alexin From this curve is obtained the value of the abscissa of the standard corresponding to the fractional amount of hemolysis produced in each tube of the unknown series, or to the 50 per cent ordinate determined by interpolation on a curve drawn through the experimental points of the unknown.

Simple calculation then gives the ratio of the hemolysin concentration of the unknown to that of the standard. The concentration of any solution in units which produce a given fractional amount of hemolysis under the conditions of each experiment may be represented by M/V where M is the dilution, or volume in cc. in which is contained 1 cc. of the serum, or protein fraction referred to the original serum volume, and V is the volume of diluted solution which is required for the given amount of hemolysis. Then the ratio of unknown concentration to concentration of standard is given as follows:

$$\frac{M_x}{M_{std.}} \times \frac{V_{std}}{V_x} = \frac{C_x}{C_{std.}}$$

for each fractional amount of hemolysis.

The ratios obtained from varying amounts of hemolysis have been found to agree within 4 per cent if the ordinates chosen lie between 15 and 85 per cent on the scale of ordinates so that the probable error or divergence from the mean is not greater than 2 per cent for any single reading. Consequently a single tube of dilution of unknown may be used for measurement of hemolysin concentration, if its value falls within the limits mentioned.

It is important that both alexin and erythrocytes be fresh and that the cells be washed in only two changes of isotonic suspending medium. More thorough washing of the cells leads to higher values of hemolysis in the first part of the curve, with little effect on the hemolysis of the final portion of the curve, so that greater error is introduced in determining the abscissa of the standard which corresponds to the ordinate of the unknown.

Comparison at different times, with different specimens of erythrocytes and alexin, between the same unknown solution and the same standard has given values that agree within 2 per cent.

SUMMARY.

A method is described for the measurement of hemolysin concentration, which makes possible exact comparison of results obtained at different times and with different specimens of erythrocytes and alexin; and gives precise values with an error not greater than 2 per cent.

THE PROTEIN ASSOCIATED WITH HEMOLYSIN IN RABBIT SERUM AND PLASMA.

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The immune substances or antibodies do not occur free in the blood, but are found associated with the proteins of the serum or plasma. With certain phenomena in the combination of immune body and antigen, the behavior commonly observed may be a function rather of the associated protein than of the immune body itself. "Protective" or "sensitizing" colloid effects on cell suspensions are brought about by a number of proteins which do not possess immune body.[1-4] The formation of a film of protein on the surface of the cell has been offered as an explanation of such cases.[1,2] It is not certain, however, that it is the protein nature of the film in the case of immune sera which is responsible for the changes in the physicochemical properties of the immune system. On account of the influence of "indifferent" proteins, and of protein derivatives,[4-6] even when present in low concentration, experiments planned to throw light on this problem can be undertaken only with immune body preparations that contain no protein except that intimately associated with immune body. The present investigation was undertaken in the endeavor to obtain such a protein fraction of immune serum or plasma. Hemolysin was chosen as the immune body because of the relative ease and accuracy with which this antibody may be titrated.

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[1] Coulter, C. B., *J. Gen. Physiol.*, 1921-22, iv, 403.

[2] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 655.

[3] Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921-22, iv, 669.

[4] Arkwright, J. A., *J. Hyg.*, 1914, xiv, 261.

[5] De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 395.

[6] Putter, E., *Z. Immunitätsforsch., Orig.*, 1921, xxxii, 538.

Hemolysin, or hemolytic sensitizer, is associated in rabbit serum with the globulin, as is immune body in general,[7] and has been found chiefly or entirely in the pseudoglobulin fraction.[8–11] Recently, however, hemolysin has been described as occurring entirely or in greater part in the euglobulin fraction.[12,13] The assignment of immune body to one fraction or another must depend on the experimental definition of the fractions of serum.

In the present work protein fractions have been obtained from the serum or plasma of rabbits immunized to sheep erythrocytes by dilution with water, and dialysis, after adjustment of the pH to the optimum for separation of the less soluble or globulin fractions. The content in hemolysin of the fractions obtained was determined by the method described in a previous paper.[14]

From serum the fraction commonly known as euglobulin was found to precipitate on dilution at an optimal value of pH 5.9 to 5.8. The amount of protein brought down and the hemolysin recovered in the reprecipitated globulin varied with the degree of dilution: the precipitate from a dilution of 1 to 5 contained 1.4 per cent and from 1 to 20 dilution contained in three sera 25, 36, and 45 per cent of the total hemolysin of the corresponding whole serum. Further lessening of the electrolyte concentration by dialysis in 1 to 6 dilution of whole serum or of the solution from which globulin had been removed by dilution alone led to the separation of a larger amount of globulin and immune body. The pH of the solutions was adjusted to 5.9; dialysis was carried out in collodion sacs for 1 to 7 days. The total amount of immune body recovered by this procedure represented 45, 28, and 62 per cent of the total present in three sera. The first value represents an increase of 9.5 per cent in terms of the content of the whole serum over that obtained by dilution to 1 to 20 alone, at pH 5.8.

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[7] Pick, E. P., *Beitr. chem. Physiol. u. Path.*, 1902, i, 351.

[8] Fuhrmann, F., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 417.

[9] Meyer, K., *Arch. Hyg.*, 1908, lxvii, 114.

[10] Ruppel, W. G., Ornstein, O., Carl, J., and Lasch, G., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 188.

[11] Locke, A., and Hirsch, E. F., *J. Inf. Dis.*, 1924, xxxv, 519.

[12] Otto and Sukeninkowa, *Z. Hyg. u. Infectionskrankh.*, 1924, ci, 398.

[13] Laubenheimer, K., and Vollmar, H., *Z. Hyg. u. Infectionskrankh.*, 1926, cvi, 202.

[14] Coulter, C. B., *J. Gen. Physiol.*, 1926–27, x, 541.

This globulin precipitated most promptly from aqueous solution at pH 5.65. No fractionation of the protein was possible by variation of the pH of aqueous solutions. If, however, NaCl in substance was added to the concentration of isotonicity to an aqueous solution of the globulin, a precipitate was obtained which varied in amount and in time of appearance in different specimens and formed in a rather wide range of pH with an optimum near pH 5.0. It was often incompletely soluble in water at pH 7.0 to 8.0.

Preparations from two sera had a hemolysin content of 0.15 and 0.63 per cent of that of the corresponding whole serum. The hemolytic activity of the globulin solution after the separation of the salt-insoluble precipitate was found to be 1, 5, and 12 per cent greater than before its removal. The salt-insoluble fraction appears thus to act as antialexin; this property is destroyed by heating to 50°C. The nature of this fraction is uncertain. It was at first regarded as a denaturation product, as described by Wu and Yen,[15] as a result of the hydrion concentrations to which the serum had been subjected. Later work with plasma suggested that it may be residual fibrinogen.

The hemolysin content of the solution which remained after dialysis was similar to that obtained with plasma, which is described below.

In obtaining plasma from immunized rabbits, coagulation was prevented by sodium citrate or potassium oxalate. On dilution of plasma with water, a flocculent precipitate consisting mainly of fibrinogen appeared at the optimal reaction of pH 6.4 to 6.1.

This fraction from a 1 to 5 dilution contained 2.3 and 2.5 per cent, and from 1 to 10 dilution 2.6, 5.5, and 6.5 per cent of the hemolysin of the corresponding whole plasma. In carrying out the titrations of solutions containing fibrinogen it was necessary to make the initial dilutions with water, to prevent fibrin formation. The fibrinogen itself in this fraction appears not to carry immune body: the hemolytic titre after the separation of fibrin by initial dilution in saline solution was the same within the limit of error in measurement, as when clotting was prevented by initial dilution in water. Furthermore, the presence of fibrinogen under certain conditions depresses the apparent hemolytic activity of the immune protein, as found and described below, with the second fraction of plasma.

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[15] Wu, H., and Yen, D., *J. Biochem.*, 1924–25, iv, 345.

Globulin and a second portion of fibrinogen were separated from the remaining plasma solution by dialysis at pH 6.1 against distilled water. Thymol was added, and dialysis carried out at 5°–10°C. for 3 to 15 days. The greater portion of water-insoluble protein flocculated in 48 hours; a further small amount, which was identical in its solubility with the first portion, separated slowly during 15 days or more. The precipitate, which appeared on dialysis, was soluble in water, and showed an optimum for flocculation from aqueous solution at pH 6.1 An hemolysin content of 33, 48.5, 48, 62, and 68 per cent of that of the whole plasma was obtained in this fraction. Longer dialysis was employed with the later experiments, with resulting increase in separation of hemolysin as indicated by the values which are given in the order in which the experiments were carried out.

The fibrinogen present in this fraction separated out as fibrin when NaCl was added to isotonicity to an aqueous solution. Fibrin formation took place at reactions between pH 8.0 and 5.6, with an optimum about pH 7.2; separation was slow and three or more clottings were observed if each clot was removed as it formed. Within the limits of pH given, the formation of fibrin gel was most rapid and complete within certain concentrations of NaCl or $CaCl_2$; the limits were not precisely determined, but approximated isotonicity for NaCl. On the acid side of pH 6.0 fibrinogen separated out as a granular precipitate which was greatest in amount at pH 5.0 to 4.8. Fractions of immune protein which contained fibrinogen showed the greatest hemolytic activity, when brought in saline solution to pH between 8.0 and 4.0, at the reactions optimal for formation of fibrin gel. The presence of fibrinogen, possibly because of the form in which it existed, in solutions at other reactions appears to depress the hemolytic activity.

The total N associated with one hemolytic unit, of the usual value, was found with globulin preparations freed from fibrinogen to be 0.00038, 0.00034, and 0.00007 mg. with three different specimens.

The values are very close to those of Locke and Hirsch[11] for hemolysin obtained by dissociation from specific combination.

The hemolysin present in the plasma solution from which water-insoluble globulin had been separated by dialysis varied inversely with the amount recovered in the globulin fraction; a minimum of 2.8 per cent of the total was found in a specimen which had been dialyzed for 15 days.

DISCUSSION.

The globulin with which hemolysin is associated appears to exist in plasma as an adsorption complex with fibrinogen; the latter determines the optimal pH of flocculation from aqueous solution of the complex. The conditions under which fibrinogen forms a fibrin clot, or gel, recall those found by Falk[16] for gel formation by banana protein; in both cases gel forms only on the alkaline side of pH 6.0 and within certain limits of salt concentration. The relation of Ca to fibrin formation in the solutions studied is not as evident as in the case of banana protein; Ca is known to be necessary for clotting only in the first step, for which blood Ca was available in these plasma preparations.

The granular form in which fibrinogen separates from isotonic NaCl solution at pH 5.0 to 4.8 suggests that a similar precipitate from serum protein solutions is residual fibrinogen. Both fibrinogen and the salt-insoluble protein from serum carry down no hemolysin or only an insignificant amount which may represent adsorbed globulin. The depression of hemolytic activity by amorphous precipitate of fibrinogen is perhaps due to adsorption of alexin.

Extraction of immune globulin preparations, with 8 to 20 volumes of 95 per cent alcohol at 0°C., caused a loss of at least 90 per cent of the immune body. The evaporated residue of the alcoholic extract contained both protein and lipoid, which neither alone nor together showed hemolytic activity. At the same time, the hemagglutinating property of the immune protein was found considerably intensified after alcoholic extraction. The partial denaturation of the protein by alcohol destroys the hemolytic activity, which must depend upon a different property of the protein from its agglutinating action.

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[16] McGuire, G., and Falk, K. G., *J. Gen. Physiol.*, 1921–22, iv, 437.

The water-insoluble globulin which was obtained by dialysis was found to contain both euglobulin and pseudoglobulin, defined as fractions precipitated by one-third and one-half saturation with ammonium sulfate. Although the separation of pseudoglobulin and immune body from serum or plasma by dialysis at optimal pH was not complete, the present work confirms the view of Pauli and Adolf[17–19] that no distinction can be made between a water-soluble and a water-insoluble globulin.

SUMMARY.

1. The water-insoluble globulin with which hemolysin is associated, may be separated from immune serum or plasma by dilution and simple dialysis at optimal pH.

2. This optimum in plasma is influenced by the presence of the fibrinogen.

3. Fibrinogen carries no immune body, or only an insignificant amount; when present in immune body solutions in other form than fibrin gel, *it depresses the hemolytic activity.* The conditions for the formation of fibrin gel are similar to those for the formation of a gel by banana protein.

4. The hemolytic activity is a more labile property of the immune protein than the agglutinating activity; hemolysin is destroyed, hemagglutinin shows an apparent increase, as a result of alcohol extraction.

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[17] Pauli, W., *Biochem. Z.*, 1924, clii, 355.

[18] Adolf, M., and Pauli, W., *Biochem. Z.*, 1924, clii, 360.

[19] Adolf, M , *Klin. Woch* , 1924, iii, 1214.

"GALVANOTROPISM" OF ROOTS.

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I.

The galvanotropic orientation of animals may probably be used for analysis of certain features of central nervous activity.[1] Therefore it is necessary to obtain a conception of the mode of stimulation by passage of direct current. In relation especially to the results of studies, in this laboratory[2] and elsewhere, upon the conductance of plant cells, and because of the structurally simpler conditions of response, we have paid attention to the well known "galvanotropic curvature" of roots.

Every one interested in plant irritability has always considered the "galvanotropic response" of the roots as one of the most remarkable facts described. Discovered by Elfving in 1882, the "galvanotropic response" was studied by Müller-Hettlingen, 1883; Brunchorst, 1884; Rischawi, 1885; Ewart and Bayliss, 1906; Schellenberg, 1906; and in a rather long paper by Gassner, 1906. Indications are given also by Szücs, 1913, and a general review of the subject can be found in Stern's book (1924).

Based on all these observations, it is generally admitted that for high densities of current, or for long exposures, a curvature towards the + pole is obtained (so called Elfving's curvature); that, on the other hand, for lower densities of current, or for shorter exposures, the curvature is directed toward the – pole (genuine "galvanotropic response"). This last is considered as a tropism, the first curvature being said to be purely traumatic.

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* Advanced fellow, Commission for Relief in Belgium, Educational Foundation.

[1] *CF.* Crozier, W. J., 1926, *J. Gen. Physiol.*, ix, 395.

[2] Osterhout, W. J. V., 1926, *J. Gen. Physiol.*, viii, 131. Blinks, L. R., 1926, The permeability and electrical conductivity of single cells, Thesis, Harvard University, Cambridge, 1926.

The definition of the true "galvanotropism" of the root can be therefore expressed in the following manner: It is a growth curvature, directed towards the cathode, located in the region of maximum growth, irreversible by plasmolysis and requiring the presence of the tip of the root.

The origin of this curvature is naturally the real question. For the explanation of this origin several theories have been advanced. Brunchorst sees in the curvature an injury by the electrolysis products, especially the H_2O_2 that may be produced. Rischawi considers the phenomenon similar to the electrosmotic water displacement in the albumin cylinders of du Bois Reymond's experiments. Ewart and Bayliss attribute the response to chemotropic stimulation by the products of electrolysis, all idea of traumatic curvature being excluded.

On the contrary, for Gassner the facts may be explained as a traumatic response of the organism to a unilateral injury of the tip of the root.

In fact, to all these explanations the same objection may be made: the technical conditions were often too crude and, in a certain number of cases, too vague to be used adequately as bases for explanations. For instance, Ewart and Bayliss used platinum electrodes directly in contact with the plant. In other cases, it is true, so called unpolarizable electrodes were employed. Others (Brunchorst, Gassner, Schellenberg) used carbon electrodes, often dipping directly in the same liquid as the root tips but sometimes surrounded by a septum made by a porous plate. For many cases the density of current corresponding to the position of the roots in the trough is not known.

It was thought interesting for these reasons to reproduce these experiments, trying always to avoid the errors pointed out.

The actual experiments were carried out keeping in mind the following points: (1) Reduction of polarization products by use of unpolarizable electrodes, (2) prevention of diffusion of the products of electrolysis by use of agar blocks, (3) gradient of densities of current easily controllable by use of troughs with definite geometrical shapes and sizes.

II.

Technique.

1. Trough 1.—Paraffine blocks were carved out following the indications of Fig. 1. These troughs are characterized by their variable cross-section, one end being a square of 5 cm. of side, the other end being 2 cm. × 5 cm. The distance of these two sections is 25 cm. and each section is closed by a block of agar gel. The agar was purified agar which had been soaked in 2 per cent HCl for 24 hours; then in 1 per cent ammonia for 12 hours; then subjected to running water for 48 hours, all with frequent shaking. After this treatment the water was more or less pressed out and the agar washed several times with distilled water, this being also used to make the gel, of which the concentration was 10 per cent in dry weight of agar. This agar gel is poured in place, care being taken to obtain a plane surface at both ends of the trough (the real electrode surfaces) and cups being provided in each block for the electrodes. These cups as well as the trough are filled to a definite height with tap water or with a balanced salt solution (diluted Knop solution). This liquid was removed immediately after each experiment and the trough was washed for several hours by a continual flow of tap water.

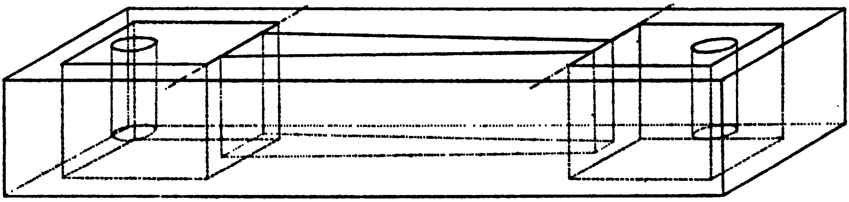

FIG. 1. The figure represents Trough 1. In Trough 2 the blocks of agar are omitted and replaced by porous plates in the vertical planes of the dot-dashed lines.

2. Trough 2.—The other type of trough is similar, the differences being only in the replacement of the agar blocks by porous plates 2 mm. thick.

3. Electrodes.—The electrodes used were carbon cylinders 2.5 cm. in diameter, a good contact being assured for the leads; or unpolarizable electrodes, either calomel electrodes (employed in a few cases only, the inner resistance being too high) or of zinc-zinc sulfate. In this last case the electrodes, which were kept in a concentrate solution of zinc sulfate, were rapidly washed before use under running tap water. Furthermore, care was taken to have the same hydrostatic level in cups and electrode.

4. *Current.*—The potential differences applied to the electrodes varied from 0.5 volts to 115 volts. The density of current (according to the position of the root in the trough) varied from 0.058 ma./sq. cm. to 1 ma./sq. cm. The time of exposure to the action of the current was changed between 15 minutes and 360 minutes.

5. *Material.*—The plants used were *Vicia faba* (broad Windsor beans) and *Phaseolus vulgaris* (Burpee's improved bush lima beans). The seedlings were grown in sawdust at 20°C. and their roots were practically straight. They were used when the length of the roots was 4 to 6 cm. In a few cases experiments were made with secondary roots with the same results as with primary ones. Normally the roots dipped for 8 to 10 mm. in the solution.

III.

Results.

1. When Trough 1 is used alone, whatever the P.D. or density of current may be, and whatever the duration of the experiment (between 15 minutes and 360 minutes) no curvature ever occurs.

When Trough 2 is used, with the same conditions, curvatures are shown if carbon electrodes are used and always towards the cathode.

A test was to put Troughs 1 and 2 in series, as the objection could be made that the density or time was deficient: under these conditions curvature appears in No. 2; no curvature in No. 1, so the current and time of exposure were large enough to produce "galvanotropic" responses.

The immediate conclusion to be drawn from these experiments is that "galvanotropic" curvature is produced by the products of electrolysis.

2. Was therefore the galvanic current necessary? Sets of seedlings were put with the root dipping for 1 cm. in the water of Trough 2, for 24 hours, after the current had been passed through the water for

2 and 4 hours. Care was taken not to move or disturb the water during or after the electrolysis.

The immediate result of this test was to show a slight but definite curvature—in fact less than when the current was acting directly on the seedlings and practically not directed toward one pole or the other. The angle of deflection of the tip of the root was about 15° to 20°.

If the same experiment was made, with the same conditions (carbon electrodes, 2 or 4 hours previous passage of current) with Trough 1, no curvature is shown.

This fact proves that the blocks of agar, 10 cm. in length, are sufficient to prevent the diffusion of products of electrolysis or to slow it up so that they do not reach the middle part of the trough in time to affect the seeds.

There is therefore a difference in the curvatures when current is present or absent but the difference is merely quantitative.

We must for this reason recognize in the "galvanotropic" curvature a double effect: the first being produced by the electrolysis products; the second being the further increase of the first under the persistence of the electrical current.

The second test, showing the influence of the electrolysis products is, in fact, sufficient to show that the primary effect on the root is a traumatic one.

3. Another way to show this was to injure the root before the experiment and to place it then in Trough 1. If really injury is the first step, curvature must occur under these conditions, with non-polarizable electrodes. And it does.

Roots were placed for 2 to 3 minutes in a solution of copper nitrate N/100; then in Trough 1 filled with tap water. The current was passed for 60 minutes. The plants were left in place in the same liquid for 24 hours (as in all the other experiments) after the passage of the current. At the end of this time a definite cathodic curvature was shown.

Experiments made after immersion of the roots for 10 minutes in the same copper solution did not give curvature.

Microchemically it is easy to show that during the short exposure the two external layers of cells are permeated by the copper ion, and that in the second case a much larger number of layers are injured. The latter injury corresponds practically to the killing of the root.

So, to produce the "galvanotropic" curvature in the absence of electrolysis products, the injury must be definite but not too large. A certain amount of tissue must remain in the root to react. The curvature is in fact a response from injured but not dead tissues.

IV.

Interpretation.

How may we try to explain these facts? As was pointed out previously the response must surely be developed in two steps (or more).

Let us consider a root dipping in the water between the two electrodes. This body in the electrical field naturally repells the lines of flow around itself, as we know that the living cells are practically nonconducting for direct current. But under these conditions there may be accumulation on the opposite sides of ions of opposite signs. And this may be sufficient to injure the epidermic layer of the root. What are the ions which are so toxic? It may be said that perhaps any ion present in the solution used will act in this way.

In a root of circular cross-section we may thus consider two opposite halves in the epidermic layer, both injured and probably with a number of the cells killed, and acting for the remaining part as two electrodes directly applied on the internal tissues. These electrodes determine, inside of the root, an electrical field, the conducting paths being made by the cellulosic membranes imbibed with aqueous solutions. The resulting electrolysis acts now on more deeply situated cells.

The produced ions are formed in such loci that they may act directly on the plasmatic surfaces, inducing in these such changes that the relative dielectrical resistance diminishes and that free ions may migrate in or out of each cell. The result is necessarily that under the directing action of the electrical field, in all cells through the root, chains of + and − charges are formed, each cell having a + charge on the cathodic side and a − charge on the anodic side. The perduration of the current continues the same action in the same way and causes finally a relative increase of anions on the anodic side and of cations on the cathodic side. The two halves of the root are brought, by this process, to be ionically different. The early effect of the anions on the anodic side seems not to prevent growth. The relative accumulation of cations, on the contrary, slows the growth on the cathodic side, and by further increase stops it completely. The effect of this differential state is a bending towards the cathode.

V.

In the earlier papers on galvanotropism another type of curvature has been described, the so called Elfving's or anodic curvature. With the device described in the present paper it has always been impossible to obtain this effect, even when the root was deeply dipping in the water. We may draw from this fact the deduction that in the cases described, with very high densities of current, other factors were interfering.

The same can be said as to the *S*-shaped curvature described by Gassner when more than the tip of the root dips into the liquid: in the present conditions of experimentation no case of this bending was found. Is it therefore to be deducted that this type of curvature does not exist? No, probably; but that under the conditions of experimentation, insufficiently described in the papers to which reference is made, other effects are occurring. Other experiments are necessary to ascertain the conditions of production of these curvatures, as well as the change in conductance of the tissues showing or not showing these reported curvatures.

SUMMARY.

1. New experiments, made in such a way to eliminate as completely as possible products of polarization and the migration of such products when formed, have shown that the exhibition of galvanotropic curvature in roots is mainly dependent upon such products, since no curvature appears when they are excluded.

2. The polarization products injure the external layer of cells of the root; this allows these cells to act as electrodes directly applied on the internal tissues. The inner electrolysis produces such changes in the interior cells that they may be considered as becoming ionically different. This differential state is responsible for curvature.

3. "Galvanotropism" of roots, therefore, cannot be regarded as exactly comparable to the galvanotropic orientations of certain animals, but is essentially dependent upon injury.

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THE FREE ENERGY OF NITROGEN FIXATION BY LIVING FORMS.

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(Accepted for publication, January 14, 1927.)

The view-point that energy is required for the fixation of nitrogen by the various forms of life seems to be generally taken for granted among biologists.

It is the purpose of this paper to present evidence of a simple thermodynamic nature against the soundness of this current conception. It will maintain that, contrary to the current view, energy may not be required for fixation, and that moreover, in fixation considerable energy may be released and placed at the disposal of the organism. In contending for a position so plainly the reverse of the old, important thermal data will be used which has been obtained only in the last decade.

This paper, as so many others, is indebted to Lewis and Randall[1] for most of the free energy values and conventions to be used. Unless it is otherwise stated, the temperature of all the reactions cited is 25°C. The heat of reaction and the free energy of reaction will be negative when heat and free energy respectively are liberated.

Nitrogen fixation may be defined as the primary step or steps in the formation of a simple nitrogen compound such as ammonia or nitrate from pure elementary nitrogen. It is to be distinguished from later changes in which ammonia and nitrate are formed one from the other, and also from the building up of higher compounds with carbon. When an organism once forms a simple compound from elementary nitrogen, the energy relations are those which would exist were it to be supplied with such a simple compound and we must discriminate between the energy of nitrogen fixation and that of nitrogen metabolism.

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[1] Lewis, G. N., and Randall, M., Thermodynamics, New York, 1st edition, 1923.

The standard free energy, ΔF°, of the reaction

$$1/2\ N_2(\text{gas}) + 5/4\ O_2(\text{gas}) + 1/2\ H_2O(\text{liquid}) = HNO_3(\text{aqueous})$$

is a small positive value, showing that at standard conditions this reaction is not spontaneous and will proceed only with the addition of free energy. At standard conditions all the reacting substances have unit activity; that is to say, the gases have fugacities of one atmosphere (for the present calculations their pressures may be considered equal to their fugacities), the water is pure, liquid, and at atmospheric pressure, and the concentration of the nitric acid is at the hypothetical 1 molal. Let us now calculate the conditions in the plant when the above reaction will proceed without the addition of free energy, that is to say, when the free energy, ΔF, will be negative. Avoiding unwieldy overrefinements, let us consider as appropriate approximations, the pressure of nitrogen, .8 atmosphere; the pressure of oxygen, .2 atmosphere; the plant sap to have the same activity as pure water; and the activity of the nitric acid formed to be unaffected by the various foreign substances in the sap.

The standard molal free energies of nitrogen, oxygen, water, and nitric acid are respectively 0,0, −56,560, and −26,500 (calories). Hence in this reaction ΔF° is 1780. Now,

$$\Delta F^\circ = -RT\, ln\, K = -1364\ 9 \log K$$

$$\log K = \log\ (HNO_3\ (\text{aq.})\ /\ (N_2)^{\frac{1}{2}}(O_2)^{\frac{5}{4}}) = -1780/1364\ 9 = -1\ 304. \quad \text{Or, } K = .0497$$

$$\text{activity } HNO_3 = K \times (.8)^{\frac{1}{2}} \times (.2)^{\frac{5}{4}} = .0067$$

From the table containing the activities of nitric acid at various concentrations this value corresponds to a concentration of just .1 M. This means that, assuming the plant can catalyze the reaction, nitrate will form in the plant with the liberation of free energy so long as its concentration remains below .1 M or 6200 parts per million by weight. At this point ΔF no longer has a negative sign but is equal to zero, since this is the equilibrium concentration.

The heat of the reaction is −49,100 − (−68,310/2) = −14,940. Owing to the fact that the plant sap rarely if ever reaches a concentration of 6200 ppm., for every mol of nitrate which forms from air and water according to the above equation there are 14,940 calories of heat (not work) liberated for partial use as chemical energy. The more dilute the concentration at which the nitrate is formed the greater is the amount of available work (not heat) placed at the plant's disposal. Since nitrate in the plant sap is removed soon after its appearance, its concentration probably never rises much above, let us say, .001 M. If we assume a maximum concentration (*i.e.* stationary state) of nitrate in the plant sap of 6 ppm. or .0001 M, and that the activity coefficient at this concentration is unity, then the following calculations show that the free energy is −7870 per mol of nitrate formed.

$$K = (.0001)^2 / (.8)^{\frac{1}{2}} (.2)^{\frac{5}{4}} = 8.6 \times 10^{-8}.$$
$$\Delta F = \Delta F^\circ + RT \ln K = 1780 + 1364\ 9\,(-7.07) = -7870.$$

Similar calculations show that after making the permissible assumption that the heat of the reaction would not change significantly under conditions of such extreme dilution, the free energy of the reaction would equal the heat of the reaction, −14,940, that is to say, the process would be 100 per cent efficient, when the concentration of nitrate was 7×10^{-6} M or .4 ppm. In the sap of some plants this is the highest concentration to be found. Indeed, in studies by Hoagland and Davis with the sap of *Nitella* cells it was found that, although the pond water in which *Nitella* was growing contained about .5 ppm. of nitrate, the cell sap contained no detectable amount. If this small concentration of nitrate results from the rapid use made of nitrate by the cell, it is evidence of how efficiently the fixation of nitrogen may be accomplished. Comparison of the free energy (−7870) at a nitrate concentration of 6 ppm. with the free energy (−14,940) at the lower concentration of only .4 ppm. shows how quickly the free energy increases as the concentration of nitrate decreases.

A citation from Lewis and Randall[2] will illustrate the significance of this reaction. "It is to be hoped that nature will not discover a catalyst for this reaction, which would permit all of the oxygen and part of the nitrogen of the air to turn the oceans into dilute nitric acid."

Although foreign to the discussion of nitrogen fixation, it might be remarked that the energy needed for the reduction of nitrates following their fixation may be supplied by radiant energy in the case of higher plants. In leguminous plants, should the nitrogen-fixing bacteria produce nitrates, they might release these into the sap where they could be carried to the leaves and reduced by sunlight at no energy expense to the organism.

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[2] Lewis and Randall, p. 568.

It will be noticed in the foregoing equation that oxygen gas is required as a reactant. Does the equation for this reason fail to serve for anaerobic organisms, such as *Clostridium*, which can fix nitrogen in the absence of oxygen? We may escape this conclusion by postulating the formation of oxygen gas incidentally to some other metabolic process; a feasible supposition in absence of data known to preclude a small but continuous production of this gas during fixation. A continuous and restricted production of the gas may occur and yet escape detection, owing to the immediate use made of it by the organism. The fact that, if such a source be supposed, the activity of the oxygen in the equilibrium constant would be reduced, would affect the conclusions at issue only as regards quantities involved.

Against the value of this possible source of oxygen as support for the main contention of this paper, it may be objected that, assuming the production of oxygen gas under anaerobic conditions, would not energy be required for its production, and so leave on hand the same occasion as before for assuming an energy requirement for the fixation of nitrogen by anaerobes?

Admittedly, an examination of the score of available free energy data of organic compounds conclusively suggests energy requirement for the production of oxygen. A striking and obvious case is found in the reaction of formic acid to yield oxygen and formaldehyde, the standard free energy having a positive value of about 60,000 calories, an enormous amount when considered relatively to the free energies of either of the compounds, which are, respectively, $-87,920$ and $-30,000$ (approximately). And in general this behavior might be expected from most if not all organic compounds. However, there might be exceptions, since such inorganic exceptions can be found. In the reaction of $HBrO_3$ to yield HBrO and O_2, 14,280 calories of heat are liberated and the standard free energy amounts to $-21,980$; and there are several inorganic reactions similar to this one, which is given merely as an example of possibility but not with a view to application. Hydrogen peroxide is another example and the yield of free energy is even larger than in the case just cited. There might be organic peroxides with a similar behavior.

However, in anaerobic processes oxygen need not be considered. The production of aqueous ammonia from nitrogen and hydrogen is attended by a negative standard free energy change of 6300 calories and a negative heat of reaction of 20,300 calories. This exothermic and free energy-yielding production of ammonia resembles that of nitric acid, and a similar correspondence holds between the effects which result from the formation and removal at high dilution of the two compounds ammonia and nitric acid.

Out of curiosity let us glance at the standard free energy which would be yielded if oxygen were present also. If the reaction proceeded according to

$$1/2\ N_2(g.) + 5/2\ H_2(g.) + 1/2\ O_2(g.) = NH_4OH(aq.)$$

rather than in the manner above,

$$1/2\ N_2(g.) + 3/2\ H_2(g.) + H_2O(l) = NH_4OH(aq.)$$

the standard free energy would be $-62{,}860$ as against the former -6300. This is a great increase, owing for the most part to the fact that oxygen of water as a reactant is replaced by oxygen gas. In the same way in the aerobic fixation of nitric acid if hydrogen were present making the reaction proceed

$$1/2\ N_2(g.) + 1/2\ H_2(g.) + 3/2\ O_2(g.) = HNO_3\ (aq.);\ \Delta F^\circ\ -26{,}500;\ \Delta H\ -49{,}100;$$

then a great deal more free energy would be evolved than in the original case discussed.

In these reactions the need for hydrogen production might be satisfied from several independent sources with no expense of free energy by the organism.

In anaerobic processes hydrogen is often an end-product incidental to metabolism, and would waste if not conserved in fixation.

In both aerobic and anaerobic metabolism many reactions, more particularly the breakdown of organic acids, may proceed spontaneously to yield both hydrogen and energy. For example,

$$HCOOH(aq.) = CO_2(g.) + H_2(g.);\ \Delta F^\circ = -6340.$$

It may be argued that the organism sustains a net potential if not actual loss of energy when the reaction proceeds in this manner, rather than by combustion, when 61,000 calories of heat are yielded; whereas in the reaction cited the heat of the reaction is approximately zero. To this objection it is enough to answer that if no oxygen were present this energy could not be yielded. But assuming an occurrence of oxygen, then since the heat of combustion is less by some 40,000 calories than the heat of formation (−101,000) there results a veritable gain of energy upon replacement of the HCOOH molecule. To object that the HCOOH molecule is not replaced under certain conditions is unwittingly to concede that under these conditions HCOOH is a waste product. Were HCOOH derived from glucose and oxygen gas, instead of from its elements, the difference between the heats of formation of $\frac{1}{6}$ mol of glucose and 1 mol of HCOOH; that is to say, the veritable gain of energy, is still larger than the value 40,000 calories by 10,000 calories. And if the glucose were derived from carbon dioxide and water, instead of from its elements, then an enormous amount of energy is required for fixation providing that (1) it is not fixed as HNO_3, (2) it is fixed as ammonia, the hydrogen coming solely from the glucose (or ultimately from the water), (3) the intermediate product is not a waste product. In terms of standard free energy, 65,900 calories are required at a pH of about 5, increasing as the sap becomes more alkaline, to a value of 78,500. The free energies would differ relatively little from the standard free energies (approximately 10 per cent). Such a reaction will be referred to later in discussing a paper by Linhart.

There are several organic acids whose heats of formation are greater than their heats of combustion, even greater than in the case of HCOOH. These acids are often the end and therefore waste products of anaerobic metabolism, not to say of aerobic metabolism. Indeed, Stoklasa[3] reports finding formic, acetic, butyric, and lactic acids in pure cultures of *Azotobacter*, an aerobic form. In these same aerobic cultures hydrogen gas was evolved.

Surprising as it may perhaps seem, an additional source of hydrogen gas is the atmosphere. According to Spoehr,[4] the atmosphere contains .01 per cent by volume of hydrogen. That this estimate is meritorious, and in addition one which varies little, is indicated by a summary of the experimental determinations given by Mellor.[5] The estimates of various investigators range from .019 per cent (Gautier) to .003 per cent (Rayleigh), the majority falling near the mean of these two values. In the following discussion the value of .01 per cent will be used. This particular value can hardly mislead since the probable variation from it has been indicated.

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[3] Stoklasa, J., *Centr. Bakt.*, *2. Abt.*, 1908, xxi, 506.

This concentration of hydrogen, instead of being small, is about equal in volume to the carbon as carbon dioxide in the atmosphere. By weight the ratio of hydrogen to carbon is 1 to 6. The corresponding ratio by weight in either proteins, carbohydrates, or fats is from 1 to 6 to 1 to 8. In bacteria[6] it is about 1 to 8. The quantities by weight of carbon and hydrogen in the atmosphere can therefore be considered equal when judged by the needs of the plant.

The following calculations will call attention, with the great suggestiveness of free energy calculations, to the potential importance of atmospheric hydrogen for plant metabolism, and in particular, for nitrogen fixation.

The equilibrium concentration of ammonia which might form from the nitrogen and hydrogen of the atmosphere, with the assistance of catalysts, is .2 ppm. in alkaline sap.

$$K = -6340/-1364.9 = 4.65$$
$$\log K = \log (\mathrm{NH_4OH(aq.)}/(\mathrm{N_2})^{\frac{1}{2}} (\mathrm{H_2})^{\frac{3}{2}} (\mathrm{H_2O}))$$
$$\text{activity } \mathrm{NH_4OH(aq.)} = K \times (.8)^{\frac{1}{2}} (.0001)^{\frac{3}{2}} = 4 \times 10^{-6}\ \mathrm{M} = .2 \text{ ppm.}$$

This value of .2 ppm. corresponds to the equilibrium concentration of .1 M for nitric acid. When hydrogen is also used to form nitric acid according to the equation

$$1/2\ \mathrm{N_2(g.)} + 5/2\ \mathrm{H_2(g.)} + 3/2\ \mathrm{O_2(g.)} = \mathrm{HNO_3(aq.)}$$

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[4] Spoehr, H. A., Photosynthesis, American Chemical Society Monograph Series, New York, 1926, 36.

[5] Mellor, J. W., Comprehensive treatise on inorganic and theoretical chemistry, London, 1922, i.

[6] Russell, E. J., The microorganisms of the soil, London, 1923, 39.

the equilibrium concentration of nitrate is .16 M or 10,100 ppm., an increase of 60 per cent.

The equilibrium concentration of bases will be raised by a greater hydrogen ion concentration while that of acids will be raised by a lesser hydrogen ion concentration. Thus in the equation

$$1/2\ N_2(g.) + 3/2\ H_2(g.) + H^+ = NH_4^+,\ \Delta F^\circ = -18{,}930$$

if the sap is assumed to have a pH of about 5, the equilibrium concentration is raised to .6 ppm., a threefold increase over that in alkaline sap.

In passing, it will reward us to suggest how this large amount of hydrogen in normal air might play a rôle in the carbon dioxide assimilation of aerobic forms. For instance, if the hypothetical assimilation reaction

$$CO_2(g) + H_2O(l) = HCHO(l) + O_2,\ \Delta F^\circ = 122{,}000$$

were to proceed

$$CO_2(g) + H_2(g.) = HCHO(l) + 1/2\ O_2;\ \Delta F^\circ = 66{,}000$$

the standard free energy required for the fixation of carbon dioxide would be halved. In this case it is important to determine the actual free energies, since the activity of the hydrogen gas is but 1/10,000 that of water. At a concentration of HCHO of 1 part per million, calculations show that there exists a negligible difference (2 per cent and 5 per cent) between the free energies and the standard free energies. For two reasons this might have been anticipated; the standard free energy values are very large; with the exception of oxygen all the substances occur to their first powers in the activity coefficient.

While we need hardly resort to the last named equation to explain carbon dioxide assimilation by higher green plants, in view of the overwhelming evidence to the contrary which has accumulated since the time of Joseph Priestley, it may well be that those organisms, such as the sulfur bacteria, denitrifiers, hydrogen fixers,[7] elementary carbon fixers,[8] and others, which reduce inorganic forms of carbon

[7] Kaserer, H., *Centr. Bakt., 2. Abt.*, 1906, xvi, 681.

[8] Potter, M. C., *Proc. Roy. Soc. London, Series B*, 1908, lxxx, 239.

without the aid of radiant energy, derive their energy not solely by the oxidation of non-carbonaceous substances with oxygen gas, but partially, at least, by the reduction of CO_2 or other substances with hydrogen gas. In the same speculative vein it may be suggested that there is some degree of identity between those organisms which can fix nitrogen and those organisms which can utilize hydrogen.

It has now been shown that nitrogen can be fixed both exothermically and with the yielding of free energy either as ammonia or nitrate in aerobic processes and at least as ammonia in anaerobic processes.

Some may think that in nitrogen-fixing organisms the simple compounds like ammonia, nitrate, and others are never produced directly, and that what occurs is always the immediate formation of higher compounds with carbon. In other words, the plant may never be able to take proper advantage of the energy yielded by simple fixation. If so, and if energy can be plainly shown to be necessary for the arrangement of nitrogen into higher compounds, then there would be great reason to accept the view now held so widely, that energy is essential for the fixation of nitrogen.

To this argument which on the surface appears to carry weight, there are several objections. The author feels it advisable to enter rather fully into these.

First, physiological chemistry supplies no evidence which makes it probable or improbable.

Second, the formation of complex compounds from the simpler takes place very generally with the liberation of energy.

Third, to suppose that complex nitrogen compounds like amino acids can be formed in no more than one step would be contrary to the present principles of organic chemistry. To say the least, a very high (if not unheard of) order of reaction would need to be supposed to bring the direct formation of these compounds in keeping with our present information.

Fourth, leaving aside the question of an energy requirement in the formation of higher compounds from nitrogen gas, it is nearly certain that energy is not essential for their formation from ammonia. Appearance in this instance must not pass for fact. The energy needed to form the higher compounds may be the sum of endothermic changes of the carbon molecules and exothermic changes of the nitrogen molecules, to use a distinction not permitted at present in talking of organic compounds. If this should turn out to be true, then to say that the average of nitrogen changes requires energy would be like saying that after an assisting engine had been fastened to the rear of a freight train and was actually aiding its motion, the freight train required power to pull the engine along. It is subsequent changes of the carbon molecule rather than of the nitrogen molecule that should involve considerable changes in energy levels, owing to there being a greater range of valence change.

Fifth, an analogy to the energy change in the formation of higher nitrogen compounds may be found in the energy change when a simple non-nitrogenous carbon compound of high energy content condenses to form a carbohydrate; and without question the average free energy change of this sort is small, and of a negative character. We have the prominent instance of 6 molecules of HCHO polymerizing to form 1 of glucose. The heat of formation of HCHO according to

$$C(\text{diam.}) + H_2(\text{g.}) + 1/2\ O_2(\text{g.}) = HCHO(\text{g.})$$

has been determined only quite recently by von Wartenberg and Lerner-Steinberg[9] to be −27,800. The heat of formation of glucose is −302,000. From this we see that about 150,000 calories are liberated during this polymerization, an enormous amount which approximates one-half the heat of formation and one-quarter the heat of combustion of glucose. The conclusions are not different when free energies are considered in place of heats of reaction. Certainly the standard free energy of HCHO would be only a trifle different from the heat of formation of HCHO, but let us assume that these are equal. The value of the standard free energy of glucose has been determined from unpublished calculations by the author to be −219,700. The calculations were made by the entropy method with the use of the very excellent specific heat data containing thirty experimental points on a temperature scale which ranged from 19.1° to 287.2° absolute, obtained by Simon.[10] Consequently in the polymerization reaction, ΔF° is about −72,000. In the plant the substances would hardly be in their standard states, but this value gives a good idea of the magnitude of ΔF when the reaction takes place in somewhat concentrated solutions. However, since there are six reactants to one resultant, the concentration of HCHO need not be very dilute before ΔF would become positive, in which case, true enough, energy would be needed for polymerization. In every instance of polymerization or condensation the effect of dilution is the same; that is, the greater the dilution the more positive does ΔF become. But the plant can very simply overcome this by removing the end-products so promptly that ΔF would be negative, and presumably this occurs. Thermodynamics cannot testify that the organism does this; but the very fact that the greater number of reactions in plants go merely with the aid of non-energy-supplying enzymes or catalysts, strongly supports the view that the plant must carry on its reactions at concentrations where free energy would not be required.

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[9] von Wartenberg, H., and Lerner-Steinberg, *Z. Ang. Chem.*, 1925, xxxviii, 591.

[10] Simon, F., *Ann. Phys.*, 1922, lxviii, 241.

Sixth, the standard free energy of formation of a higher compound of nitrogen, carbon, hydrogen, and oxygen is known. For urea, ΔF° is $-47{,}280$. Thus a great deal of free energy is yielded in the formation of this higher compound. When urea is formed, not from its elements, but from two simple compounds, as in the equation

$$CO_2(aq.) + 2\,NH_3(aq.) = urea(aq.) + H_2O(l)$$

ΔF° is 5030, indicating that at standard conditions free energy is required. However, so long as urea is removed rapidly enough, then no matter how dilute the concentration of the reactants, the reaction will require no free energy. This particular reaction is typical of the reactions which ammonia may undergo with another compound to lose water, and shows that probably the standard free energy change in such condensations is small. Yet even assuming that this reaction when it occurs in a plant is at such a concentration as to require free energy, the free energy required in that event might well be drawn from a supply incidentally provided during the fixation of nitrogen into ammonia, and so the total free energy expended still remain negative.

Returning to the thermodynamic argument, there are other reactions for the free energy yielding fixation of nitrogen. HCNO (aq.) has a standard free energy of $-29{,}000$; urea, $-47{,}280$; and HNO_2 (aq.),

$-13{,}070$. These compounds may spontaneously form on condition that hydrogen gas is a reactant. Were the hydrogen, and therefore part of the oxygen, derived from water, as in the case of nitric acid discussed originally, then nitrous acid would be the only compound *not requiring* free energy for its formation, and this would be the case *only* at quite high dilution. In the cases of C_2N_2(g.), CNI(s.), HCN(aq.), NO(g.), NOCl(g.), NO_2(g.), N_2O_4(g.), and N_2O(g.), the standard free energies are positive. Accordingly, energy would be required if nitrogen is fixed as hydrocyanic acid, or as any of these substances. Since for assimilation, however, these fixed forms would be changed by reacting with water, to either an ammonia form or a nitrate form, the net result would be a yielding of free energy. For instance, in the reaction

$$\mathrm{HCN(aq.)} + \mathrm{H_2O} + 1/2\ \mathrm{O_2(g.)} = \mathrm{NH_3(aq.)} + \mathrm{CO_2(aq.)}$$

ΔF° is $-73{,}510$. The standard free energy of formation of HCN is 27,520, consequently the net result of fixation is $-45{,}990$.

It would be profitable to consider a fixation reaction which has been the source of some confusion. The reaction for the hydrolysis of nitrogen by water,

$$\mathrm{N_2(g.)} + 2\ \mathrm{H_2O(l)} = \mathrm{NH_4NO_2}$$

has a positive standard free energy of 85,690. Lewis and Randall (1923) have calculated from this that a pressure (fugacity) of nitrogen of about 10^{51} atmospheres would be required for ammonium nitrate to be formed at an equilibrium concentration of 10^{-6} M. G. Oddo[11] had measured (1915) the ionization of water in air. He found it surprisingly large and concluded that a considerable amount of NH_4NO_2 should be formed by the hydrolysis of nitrogen. Calculations show that he confused mechanism with equilibrium. Even were the water completely ionized, and $2\ H^+ + 2\ OH^-$ substituted for $2\ H_2O$ in the above equation, the pressure of nitrogen required would still be 10^{23} atmospheres. Linhart,[12] who was among the first to apply free energy data to biological problems, attempted to determine the efficiency of nitrogen fixation by *Azotobacter*. From analogy with somewhat related compounds, he made an elaborate calculation of the standard free energy of mannite, which was at that time unknown, even approximately. In seemingly arbitrary fashion he used the above highly endothermic and free energy demanding hydrolysis reaction (except that NH_4OH (aq.) was the resultant). While the free energy data of nitric acid and ammonia may not have been available at that time, the heats of reactions as approximations might have been considered. Of all the possible fixation reactions he may have correctly chosen the one employed by *Azotobacter*. This hydrolysis reaction possesses a large positive free energy because oxygen gas is not among the reactants. Falk and McGee[13] have obtained evidence that this hydrolysis reaction takes place to some extent in the presence of metallic iron, which they say they believe supplies the energy. Their evidence is not very extensive. The reaction goes appreciably in the electric arc, since only a few volts are needed.

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[11] Oddo, G., *Gazz. chim. ital.*, 1915, xlv, I, 395.

[12] Linhart, G. A., *J. Gen. Physiol.*, 1919–20, ii, 247.

There is experimental confirmation of the contentions in this paper. Workers on the nitrogen-fixing bacteria have themselves hesitated to deny that the wide ratio (of at least 1 to 50) of the nitrogen fixed to carbohydrate available might be narrowed were it possible to provide the organisms with more favorable conditions of environment. While experiments in general have not suggested that this ratio may be narrowed, Truffaut and Bezssonov[14] now present evidence that in the presence of nitrogen-fixing bacteria corn develops normally and reaches maturity in mediums devoid of organic nitrogenous matter, and that the ratio of the carbonaceous material excreted by the roots to the nitrogen fixed approaches 1 to 1. Some of the carbonaceous material would certainly serve to supply the carbon requirements of the organisms, in this way reducing the amount available for the supposed energy need in nitrogen fixation. And so, it is not at all obvious that the carbonaceous excretion functions as a source of energy. Since the carbonaceous excretion would contain about 40 per cent carbon, the ratio of excreted carbon to nitrogen fixed is only narrower. It is to be noted that in these experiments, in which according to the authors the fixation was probably done by anaerobic organisms (*i.e. Clostridium*), no sugar or carbohydrate excretions could be detected, but only organic acids (*i.e.* malic). The organic acids are precisely the substances some of which yield hydrogen with the liberation of free energy, as would be required were the equations presented in this paper to be employed.

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[13] Falk, K. G., and McGee, R. H., *Chem. and Metal. Eng.*, 1923, xxix, 224.

[14] Truffaut, G., and Bezssonov, N., *Sc. Sol*, 1925, iv, 3–53.

There is strong evidence[15] that *Azotobacter* fixes nitrogen as ammonia.

Still more relevant is the recent extensive evidence of Christensen-Weniger.[16] He finds that the process of fixation by the nodule bacteria of several species of legumes is exothermic or nearly so. The energy requirements of the nodule bacteria were not met with by increased assimilating powers in nitrogen-fertilized legumes. Whether some of the nitrogen was supplied as fertilizer or some by fixation, the final dry weight was almost the same and from the small excess of growth in the fertilized plants he was able to fix the upper limits of the energy supply. He found the eventual requirements for the nodule bacteria so small as to be unimportant. He was quite aware of the exothermic heat of formation of ammonia.

If the nitrogen were fixed by the bacteria as nitrate, which might then be reduced in others parts of the host plant by means of radiant energy, this might in a measure explain the seemingly less efficient assimilation of nitrogen by the nodule organisms in pure culture, since there they would need more energy to reduce the nitrate.

SUMMARY.

Fixation of nitrogen even with liberation of energy or free energy, will take place if either oxygen gas ,or hydrogen gas, or other substances, especially gases, whose standard free energies are close to zero, are involved to form either nitrates, ammonia, or cyanide, not to speak of still other compounds. It has been pointed out that there are two and only two general conditions where nitrogen fixation can require energy. These are, first, if nitrogen reacts with some compound like water with an already high negative free energy of formation and where negligible oxidation of nitrogen would occur; second,

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[15] Kostyschew, S., Ryskaltschuk, A , and Schwezowa, O., *Z. physiol. Chem.*, 1926, cliv, 1.

[16] Christensen-Weniger, F., *Centr. Bakt.*, *2. Abt.*, 1923, lviii, 41; *Chem. Abstr.*, 1925, xix, 2509.

if the plant does not take advantage of working at concentrations where the process would yield free energy.

If nitrogen fixation is exothermic and free energy-yielding, how is the carbohydrate requirement of nitrogen-fixing organisms to be interpreted? Are the experimental determinations of the carbon to nitrogen ratio purely circumstantial? Is further hope given to those who may experimentally try to narrow this ratio to where the carbon used is only for the carbon requirements of general metabolism, exclusive of fixation? Do not hypotheses concerning the fixation of nitrogen in the evolutionary process, which are based on the conception that energy is required, lose some of their significance? Does it not suggest that perhaps fixation is far more universal than is supposed among living forms, particularly among the higher green plants, and thereby give encouragement to those who may wish to demonstrate this experimentally? Does it not indicate that perhaps the function of fixation is often to obtain energy for use in general metabolism? Is the general carbohydrate metabolism of the fixation forms to be regarded as being merely extremely inefficient? Or most suggestive of all, is the carbohydrate serving some unobserved function?

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STUDIES ON PERMEABILITY OF MEMBRANES.

I. INTRODUCTION AND THE DIFFUSION OF IONS ACROSS THE DRIED COLLODION MEMBRANE.

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INTRODUCTION.

When the different investigations on membrane potentials are taken into consideration, it seems that the theory of these potentials may be treated in three different ways. We may distinguish (*a*) phase boundary potentials, (*b*) mixed electrode potentials, (*c*) diffusion potentials.

The first theory assumes that the potential difference of each side of the membrane against the adjacent solution consists of a jump (a sudden, almost discontinuous fall or rise) of the potential at the boundary of two phases, the liquid being the one phase and the substance of the membrane acting as solvent for electrolytes being the other. The differences in the tendency of distribution of each single kind of ion between the two solvents which are in conflict with electroneutrality, produce the potential differences. This theory, first developed briefly by Nernst(1) and thereafter thoroughly elaborated theoretically and experimentally by Haber(2), has been applied to biological membranes by Beutner(3). The latter author also found different models in which he assumes the same mechanism, and at the same time, assumes that no essential part of the P.D. observed is produced within the membrane itself. He could reproduce membranes the potential of which depends on the concentration of any cation dissolved in the adjacent solution, and does not depend only on the concentration of one particular ion, as is the case with metallic electrodes and with the glass membrane, at least in certain kinds of glass such as Haber and

Klemensiewicz(4) used in their investigations. Beutner's assumptions meet the following difficulties. In general, it can be shown that a membrane consisting of a water-immiscible phase and behaving as a solvent for the dissolved substances, cannot give any appreciable "conconcentration effect," that means: any appreciable P.D. when placed between two solutions of one electrolyte in different concentrations(5). In order to explain that such effects are brought about none the less, Beutner has to assume that in the oily phase there is a small amount of a weak acid (*e.g.* a trace of salicylic acid in salicylic-aldehyde), which is scarcely soluble in water, and that such a weak acid dissociates in the oil very strongly, like a strong acid or a salt, so that the reaction (written in terms of salts, not of ions)

$$\text{salicylic acid} + \text{KCl} \rightleftarrows \text{K salicylate} + \text{HCl}$$

will proceed amply in the direction →, while it is known that this reaction in aqueous solutions practically goes on only in the direction ←. This assumption is necessary for Beutner's theory, but the experience of the last decade does not at all confirm such a behavior of weak acids in oils.

The second theory may be applied in such cases where the membrane has the character of an electrolyte-like substance (*e.g.* silicates, such as glass, permutit). One of the ions of the membrane substance may be an inert or "colloidal" ion such as the silicate ion, while the other ion which happens to be combined with the silicate (*e.g.* Na) shows what may be called an electrolytic tension towards the solution, like a metallic electrode. This ion, which forms a component of the solid silicate, may be partially exchanged for another ion present in the solution (*e g.* H^+). Then the silicate behaves like a mixed Na and H electrode. The potential difference will depend on the kind and concentration of any such ions which are combined or are able to combine with the silicate in exchange for the ion originally combined with the silicate ion. Recently Horovitz (6–8) showed that this mechanism holds for the membrane potential produced with certain kinds of glass.

A third theory attributes the potential differences produced by membranes to the difference in the velocity of the single ions diffusing across the membrane. According to this theory, there is no abrupt change in the potential at the boundary of the membrane but a gradual change of the potential from the one boundary towards the other, as is the case with a liquid junction potential when two different electrolyte solutions are in direct contact with each other, and by diffusion or convection a zone of gradual transition between the two solutions is interposed. So, this third theory is that of a diffusion potential. In a previous paper(9) the theory of such membranes as dried collodion or apple skin was developed on this basis.

It does not follow that these three theories are contradictory to each other. It may happen that two or even all three of these possibilities are combined in the same membrane. For instance, Beutner will probably not exclude the possibility of a diffusion potential within the membrane when it is in contact with two different solutions at the two sides. He only assumes that this diffusion potential plays quantitatively a negligible part in the total observed potential difference. On the other hand, Baur(10) endeavored to prove that the diffusion potential plays the predominating part and the abrupt change of the phase boundary potential may be neglected. Cremer(11), who was perhaps the first to emphasize the importance of these considerations, left it undecided which of these sources of potential differences plays the important rôle, emphasizing rather more the diffusion potential. The difficulty of the theory of diffusion potentials seemed to be that under any known condition the diffusion potentials, except the ones produced by acids of alkalis, are not great enough to give such a great effect as the membranes sometimes do. The differences in the mobilities of the different kinds of ions, except H^+ and OH^- ions, are not sufficient. We shall show, however, that in certain membranes these differences are enormously greater than the well known small differences in aqueous solutions. It was the aim of the author (9,12) to show that for a membrane such as collodion(12,*d*) the theory of diffusion potentials is sufficient to explain all known facts. The theory of mixed electrodes need not be considered because the chemical nature of collodion (and of some lipoid membranes, such as the wax in apple skin) excludes this possibility. The substance of this membrane is not an electrolyte-like material nor does it consist of a cation and anion like a glass. However, the question may be asked whether the phase boundary theory may not be applied for collodion.

Now, for the usual permeable collodion membrane with its large pores, obviously the whole membrane effect is due only to these pores. There can be no doubt that in any of the well known dialysis experiments the dialyzing salts will go through the pores of the membrane and will not go through the substance of the collodion itself to any appreciable extent. Again, the transition from the usual large pored collodion membrane to the dried membrane is quite gradual. So it seemed most probable that the effect of the dried collodion membrane is due to the same mechanism as that of the usual collodion membrane. The dried membrane is to be regarded only as a practically obtainable limiting type of porous membrane with decreasing pore sizes.

Since the so called Donnan membrane potential has played a great part in researches on membranes in the last decade, it seems necessary to explain the relation of the Donnan potential to the different kinds of potential mentioned above. The difference is the following. All of the three mentioned theories attempt to trace the course of the potential from the one side of the membrane to the other. Two of the theories assume a more or less abrupt change of the potential at each boundary but no change elsewhere. The other theory assumes a gradual transition of the potential from the one boundary to the other. The total potential difference is made up by the sum of the single abrupt changes, or the integral of the single differential changes, according to the assumption. The Donnan theory is not concerned at all with the course of the potential across the membrane; it only takes into consideration the difference between the two sides of the membrane, and it can be applied to any case where one kind of ion, which is present only on the one side of the membrane, is permanently not present on the other side. Donnan stated as the necessary condition for the possibility of such a case occurring the non-diffusibility of one kind of ion across the membrane, *i.e.* the lack of any mobility across the membrane. However, the same condition may be the limiting case either for very low mobility of this particular ion in the membrane, or for a very low solubility of this ion in the membrane. In either case the liquid on the other side of the membrane remains permanently free from this particular kind of ion and the conditions of the Donnan equilibrium are fulfilled. In such a case, where either the mobility or the solubility of an ion within the membrane is really zero, there is an abrupt change of the potential at the boundary of the membrane. However, the Donnan theory does not consider the course of the potential at all, and it deals only with the difference of the potential between the two sides. Neither does the Donnan theory concern itself with the mechanism of the impermeability of this particular ion. It holds at least for the limiting cases of two quite different mechanisms: insolubility and immobility. In reality, however, not only do the limiting cases occur, but also cases where either the solubility or the mobility of the ion in the membrane is only diminished and not completely abolished. Thus our system includes the Donnan potential as a limiting case among different possibilities.

It is also possible to develop the theory of the porous membrane upon the assumption of *different phases.* Certainly, the solubility, the activity, the osmotic pressure of any dissolved substance, and the vapor pressure of the solvent, are different in the bulk of the solution from those obtaining within the capillary pores and channels. One could speak, therefore, of a coefficient of distribution or partition, between the bulk and the capillary space, of any particular substance which is a common component of both. However, such an attempt would lead to great difficulties and is of no advantage whatever. The capillary spaces should not be considered as phases separated by a sharp boundary from the bulk phase, but one should rather conceive the capillary spaces as being analogous to the surface layer of any phase in contradistinction to the bulk. Thus the entire surface of all the capillaries of a sieve-like membrane is an enormously enlarged surface of the solution. We emphasize this idea in order to avoid a discussion as to whether the theory of phase boundary potentials ought to be applied to sieve-like membranes.

The problem of the potential differences produced by membranes between two solutions is closely connected with the problem of the permeability of the membrane. In the various attempts to apply the different theories to the selective permeability of the membranes of living cells, the conception of a lipoid membrane as a solvent had been chiefly used, because of the influence of the well known studies of Overton. Here the membrane was assumed to be a homogeneous phase with sharply defined boundaries interposed between the inner phase (the protoplasm) and the solution in contact with the exterior of the cell. But since this theory was not satisfactory for every case, the idea of a mosaic membrane has been suggested by Nathansohn (13). This is supposed to be a porous membrane, a sieve consisting of a framework of a lipoid the pores of which are filled by an aqueous solution. Here we have the sieve-like membrane which only differs from the collodion membrane insofar as the substance of the framework is not quite inert but participates in the permeability for certain substances which are soluble in it. In any case, in order to separate these two alleged effects of the mosaic membrane we must first study the effect of a sieve membrane with an inactive framework in which the whole problem of permeability depends on the pores. For that purpose the dried collodion membrane seems to be the almost ideal model, because no other porous membrane of sufficient mechanical resistance (clay, etc.) can be obtained so easily with pores as small as in collodion. M. Traube's copper ferrocyanide membrane suffers from the lack of mechanical strength and requires always the presence of the membrane-forming substances, $CuSO_4$, on the one side and Na ferrocyanide on the other. This complication makes it almost impossible to study the properties of such membranes with those methods which proved the most suitable and simplest in the case of collodion membrane. Collander(14) has recently made a very exhaustive study of the copper ferrocyanide membrane.

1. The Problem of Direct Diffusion Experiments.

In a series of experimental studies on the dried collodion membrane (12, *d*, *g*, *h*, *i*) it has been shown that this membrane is considerably less permeable for anions than for cations. A first attempt at a theoretical treatment of the properties of such a membrane has been made in a previous paper in this journal (9).

The assumption of a relatively small permeability for anions was founded at the outset of these studies upon the following interpretation of the E.M.F. of concentration chains. When two solutions of one electrolyte in different concentrations are separated by a dried collodion membrane, a potential difference is established which in the best cases reaches the theoretical maximum value of an ordinary concentration chain with electrodes reversible for the cations. The simplest interpretation of this effect was the assumption that the anions are not, or at least are much less, mobile in the pores of the membrane than the cations. Such an assumption must, of course, be proved by direct diffusion experiments. These diffusion experiments are the subject of this paper.

In some of the previous papers (12, *d*, *i*) diffusion experiments have already been described which fairly well confirmed the assumption, but these experiments have not been perfectly satisfactory. The difficulties consisted in the following circumstances. In the first place, when the membrane was made thick enough to resist the mechanical strain in such an experiment, the time required to yield quantities of the diffused ions sufficient for chemical analysis was very long, weeks, even months. Very often the membrane did not retain its original properties for such a long time, and many experiments were spoiled in this way. On the other hand, when the membrane was made thin enough to allow a sufficient diffusion in 1 or a few days, the lack of mechanical resistance spoiled many experiments. The membrane became leaky and the leaks could be shown sometimes simply in the ordinary macroscopic way; sometimes the leaks were relatively small so that they were not manifested macroscopically, but produced a potential difference between two KCl solutions, 0.1 and 0.01 N, much smaller than the expected maximum value. This value is, theoretically, as has been shown (9), 55 millivolts, and good membranes, give, in fact, a potential difference of 50 to 53 millivolts. Leaks not large enough to be visible macroscopically become manifest by a drop of this potental difference sometimes down to 25 millivolts or much less. Nevertheless, selecting the good experiments, it could be shown, that HCl diffuses against pure water across a membrane extremely slowly, while HCl and KCl exchange cations relatively quickly across the membrane. Other arrangements for diffusion experiments gave similar results. But all of the results so far obtained are only qualitative and not really satisfactory. In order to obtain reliable results a kind of membrane had to be employed which was permeable enough to give a measurable amount of diffusion in a few days, and which, on the other hand, retained its great difference of behavior towards cations and anions, and which also retained its properties during the time of the experiment. The authors finally succeeded in obtaining the required kind of membrane by selection of a suitable kind of collodion and method of making the membrane.

2. The Method of Preparing and Standardizing the Membrane.

The general method of preparing this kind of membrane consists in pouring some solution of collodion into a cylindrical glass vessel, letting a film of collodion form adhering to the glass wall, permitting it to dry to a certain degree, and then, in distinction to the way in which the ordinary collodion membrane is made, not to detach it from the wall by means of wetting it with water, but simply by means of forceps or the fingers. The drying has to proceed rather far to allow this detachment. The best way is to wait until the detachment begins spontaneously. After the membrane has been pulled out of the glass vessel it must be dried further, for at least a day, in the open air. Several variations in this method are possible and we shall describe the one which seemed the most convenient.

When different kinds of collodion, even when dissolved in the same solvent medium, are employed, the properties of the membranes may vary to a great extent. The different samples of membranes made up from the same collodion solutions behave, though not completely, relatively fairly uniformly. As the best method of characterizing such a membrane the measurement of the potential difference between an 0.1 and an 0.01 M KCl solution separated by the membrane may be recommended. This may be called the *concentration potential* or the *Co P* of the membrane (12, *i*). This *Co P* depends to a considerable extent on the kind of collodion used. At least ten different samples of collodion were used, such as "parlodion," "commercial gun cotton No. 1," and several samples of nitrocellulose of different nitrogen content. None of these gave really good results. Some of them gave at times membranes with a relatively high *Co P*, such as "gun cotton," but were so poorly permeable (which could also be shown by the very poor electric conductivity in an aqueous solution of some neutral salt) that they were useless for diffusion experiments. Others had a better permeability but had such a low *Co P* (between 25 and 40 millivolts) that the required specific properties of the membranes were not obtained. Such membranes showed, in fact, only relatively small differences in permeability for anions and cations. For instance, an 0.1 N solution of HCl did not diffuse into pure H_2O across such membranes much slower than it did into a KCl solution. The one sort of collodion which was found satisfactory for these purposes was "Celloidin-Schering." Membranes may be made up either in a flat form, which will be more fully discussed in a subsequent paper, or in the form of bags. For diffusion experiments the bag form seemed to be preferable on account of the greater surface, though for many other purposes the flat membrane is preferable. The following method turned out to be most suitable for the required purposes.

5 gm. of commercial celloidin shreds, Schering, previously dried in the air or by short washing with absolute alcohol, are dissolved in a mixture consisting of 75 cc. of absolute alcohol and 25 cc. of anhydrous ether. The process of solution may require several days and is accelerated by frequent gentle shaking.

Suitable tubes, such as 50 cc. round bottom centrifuge tubes, are filled with the above collodion solution, permitted to stand covered until the air bubbles rise to the top and disappear, after which the greater portion of the collodion is poured off leaving about 5 cc. in the tube. This residual amount is then carefully distributed in as uniform a layer as possible by slow rotation and warming in the palms of the hands. After several minutes of such rotation and evaporation of the solvents, the tube is placed in a clamp in an inverted position, mouth downward, and permitted to drain. As stated above, it is best to leave the tube undisturbed until the membrane begins to detach spontaneously from the glass. This may require under various atmospheric conditions 2 to 4 hours. The rim of the membrane is now cut with the point of a knife and it is removed by careful and gentle pulling. Special care must be exercised not to exert too great a strain upon the bottom of the membrane, so as not to stretch it too much at that place. At this point the membrane is still quite elastic, and the distortion due to the manipulation of detachment from the glass may be corrected by gently blowing into it several times during the next 5 to 10 minutes. The bags are now placed upon a clean surface and allowed to dry in free air for about 1 or 2 days.

It will be observed that at the end of this drying period all membranes show a greater or lesser degree of shrinking and wrinkling. The wrinkling can be partially prevented by repeated blowing during the process of the drying. The degree of this shrinking appears to depend upon the extent of drying previous to the removal of the bag from the glass tube, and upon the thickness of the collodion layer. As a rule longer drying within the glass tube and the thinner layers yield the smoother membranes. It may be noted, however, that a moderate degree of shrinking does not impair a membrane for the purposes described. A membrane which is entirely smooth is as a rule not very resistant to mechanical strain. On the other hand, strongly shrunk and wrinkled membranes may be unserviceable because of lack of uniformity of shape and texture. But the characteristic properties of permeability, conductivity, in a given electrolyte solution, and of the concentration potential seem to depend to only a small extent upon the differences in the amount of shrinking and on the relative shape. It is true that the degree of shrinking is not entirely without effect. Recently Liesegang (15) reported that collodion membranes, when prevented from shrinking during the process of drying, are much more permeable than the shrunk ones. This fact has been known to the authors for a long time, though it has not been expressly published, since the interest was directed towards obtaining the limiting case of membranes with the narrowest pores possible. It has also been known to the authors, *e.g.* that a collodion membrane made by impregnating a filter paper bag (extraction shells of Schleicher and Schüll, or simply filter paper) is much more permeable than the membrane without a skeleton, even when completely dried. The *Co P* of such membranes never exceeded about 40 millivolts. For that reason this kind of membrane was abandoned by the authors, though it may be useful for other purposes.

A completely dried membrane is a perfect electric insulator and is highly electrified by gently rubbing it against the hair. Not being easily wetted by water, it retains its electric charge a very long time, even in a humid atmosphere. This property of an electric insulator may be emphasized because it shows that the subsequently described properties of permeability and electric conductivity are due to the pores and their contents and not to the chemical or physical nature of the solid nitrocellulose substance.

A quantitative expression of the most important characteristic property of these membranes is obtained by measuring the concentration potential (*Co P*) between an 0.1 N and an 0.01 N KCl solution separated by the membrane. A suitable arrangement for this measurement is shown in Fig. 1. The advantage of KCl solutions is chiefly in the lack of any diffusion potentials against the solutions of the calomel electrodes. As was stated above the best membranes will show a *Co P* at room temperature of 50 to 55 millivolts. This *Co P* may be reproduced on the same day for any membrane within a fraction of a millivolt, and within a longer period of several days or even weeks, the *Co P* of the same membrane will not vary as a rule more than 3 or 4 millivolts. The best way of preserving the membranes is to immerse them in and fill them with distilled water.

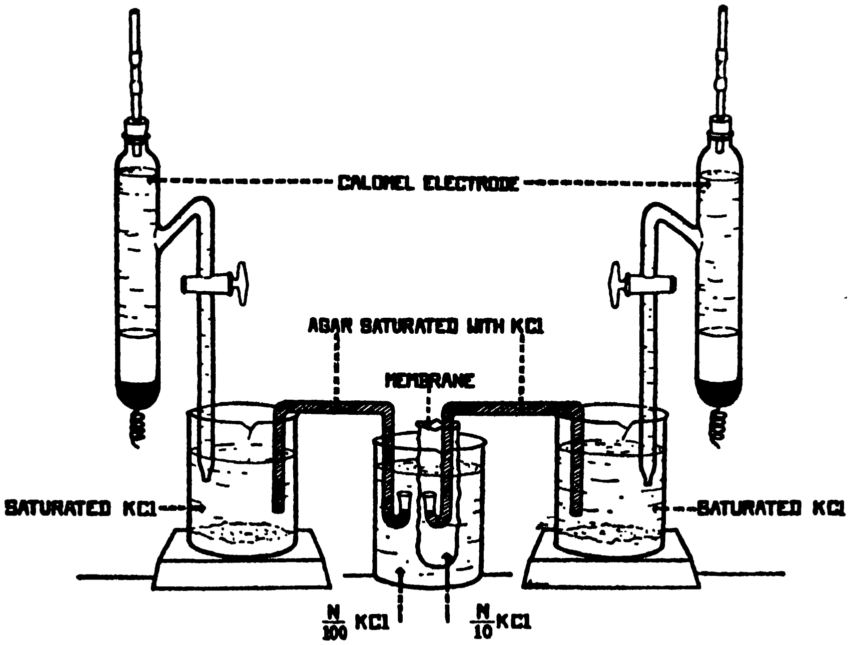

FIG. 1.

Although the electric conductance of the membrane immersed in a solution of electrolytes does not come strictly within the scope of this paper, we cannot avoid touching upon this subject. The resistance of such a system is different, of course, in the direct and alternating currents for well known reasons. The resistance which interests us from the standpoint of the permeability of the membrane for ions is the ohmic resistance to a direct current, since the magnitude of the conductance in a given electrolyte solution is in the sense of Nernst's theory a certain function of the diffusibility of the ions.

The conductance of a membrane may be approximately measured in the following simple manner using the arrangement shown in Fig. 1. The entire system is first made up *omitting the membrane*, filling the beaker with 0.01 N KCl. Now the potential difference of the system between the electrodes should be equal to zero. Being assured that such is the case, we establish by means of the potentiometer a certain potential between the electrodes of such magnitude as to produce currents of a given strength, *e.g.* 1.5×10^{-7} amperes. The current intensity is measured by means of the galvanometer, the sensitivity of which had been established by previous calibration. From the current intensity, I, and the applied E.M.F., read from the potentiometer, the resistance, R, may be easily calculated using the equation

$$I = E.M.F./R$$

For instance, with our Leeds and Northrup enclosed scale and lamp galvanometer, a deflection of ten lines on the scale indicated a current of 1.5×10^{-7} amperes. The resistance of the whole system without the membrane with a 0.01 N KCl solution in the beaker was about 3000 ohms. On inserting the membrane this resistance was increased by 600 to 8000 ohms or more, depending upon its relative smoothness and thickness.

It was surprising to find that such relatively high conductance was compatible with such highly pronounced specific influence on the relative mobilities of the cations and anions. To produce such differences in the mobilities, as are described later in this paper, we must assume extremely narrow pores. The high conductance of the membrane indicates that the number of these fine pores must be very great. Thus, for instance, for membranes prepared from a certain brand of gun cotton and which yielded about the same *Co P* effect, the resistance, under the same conditions, was found to be enormously greater, up to several million ohms, showing that in such membranes the number of pores was correspondingly smaller, while the size of the pores upon which the *Co P* depends is probably the same. On the other hand, we have observed membranes prepared from other kinds of collodion, *e.g.* parlodion, yielding much lower *Co P* values (about 25 to 30 millivolts) with a higher resistance than in the celloidin membranes. Such membranes probably have larger but fewer pores. Hence the usefulness of our celloidin membranes is probably due to a large number of very fine pores. The size of these pores is apparently within the size order of larger single molecules, for, as shown in a previous paper (12), non-electric molecules in which hydration and electric charge do not play an appreciable rôle, diffuse through these membranes at a rate corresponding to their size. For instance, urea diffuses relatively readily, while the sugars do not diffuse at all, and with aliphatic alcohols the rate of diffusion decreases rapidly with increasing molecular size.

3. *Methods of Analysis.*

The methods used were: Potassium was determined by the method of Kramer and Tisdall (16) which was slightly modified for our purposes as follows: After suitable evaporation the whole or an aliquot portion of the solution containing about 0.3 to 0.05 mg. of K in a volume of 2 cc. was precipitated with an excess (2 cc.) of the cobalti-nitrite reagent, centrifugalized after standing for several hours, the precipitate collected on a small asbestos filter, and washed on the filter several times with a dilute (approximately 0.01 M) magnesium sulfate solution. This solution was used to prevent the colloidal dispersion of the precipitate which occurs on washing with distilled water. The asbestos mat and precipitate were quantitatively removed to a small flask and titrated with N/50 permanganate and oxalate in the presence of an excess of sulfuric acid as given in the original method. Frequent checks showed that with this procedure we could determine with an accuracy of about 2 to 5 per cent analogous quantities of K in known solutions of KCl, in the presence or absence of the other salts used in our experiments.

The chloride ion was determined by the well known Mohr method using the following procedure. Since the amounts to be determined were almost always extremely small, the solutions were cautiously evaporated to dryness and the residue dissolved in 1 or 2 cc. of a 10 per cent K_2CrO_4 solution. (The chromate solution had been previously treated with a little $AgNO_3$ solution to remove traces of chloride present, and after standing for a day or 2, filtered.) The titration was carried out with N/100 $AgNO_3$ solution using microburette with a fine tip. The end-point of the titration, the first appearance of a clear brown tint, is best observed when the titration is performed in a white porcelain dish or in a glass vessel standing directly on a white surface. The excess of $AgNO_3$ necessary to produce this perceptible red brownish shade was found to amount, under these conditions, to about 0.02 to 0.05 cc. (a small drop) of the 0.01 N solution, which amount must be subtracted from the titration figure.

4. *The Diffusion Experiments.*

Several groups of experiments were carried out as follows:

I. 0.1 M KCl against H_2O Table I, A.
0.1 M KNO_3 " 0.1 M NaCl, Table I, B.
0.1 M KCl " 0.1 M $NaNO_3$, " I, C.

II. The same experiments were carried out, but in this case each membrane was used for repeated diffusion experiments with increasing periods of time of diffusion. The solutions outside and inside the collodion bag were renewed at the end of each period, Table II, A and B.

III. In Table III are detailed experiments using the same salts as above but in much higher concentrations, 0.5–1.0 M.

IV. Three experiments were carried out in which the membranes separated 0.1 M electrolyte solutions with the cation (K^+) in common but with different anions (Cl^- and NO_3^-), as shown in Table IV.

V. Table V shows a series of experiments in which the molar concentrations of the two electrolytes separated by the membrane were deliberately chosen in the ratio 1:5 with the chloride having the higher concentration. In this arrangement the P.D. of the Na^+ solution against a K^+ solution is almost completely abolished, as will be more fully explained in the discussion of the results.

VI. The experiments detailed in Table VI are presented to show the effect of low *Co P* values of the membranes and also to show that some kinds of collodion are not suitable for the purposes of approaching the characteristics of an ideal semipermeable membrane for ions. The membranes used in Series I to V were prepared for Celloidin-Schering, whereas the membranes used in this series were made from other commercial brands of nitrocellulose.

TABLE I.

Membrane No.	*Co P* of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl	Diffused K per day (24 hrs.)
	Before	After		K	Cl		
	mv.		*hrs.*	*mols* × 10^{-6}			*mols* × 10^{-6}
A. Diffusion of 0 1 M KCl against H_2O							
1	53.8		24	2.0	4.8	0.4	2
		42.0	42	1.6	3.5	0.5	0.92
2	51.5		70	5.6	5.1	1.1	1.3
3	49.8	46.7	70	3.6	3.2	1.1	1.2
B. Diffusion of 0.1 M KNO_3 against 0.1 M NaCl							
1	53.0		24	9.1	1.8	5	9.1
2		37.5	42	23.8	3.2	7	13.6
3	52.0		24	20.3	2.0	10	20.3
4		49.2	42	36.4	3.0	12	20.8
5	52.0	54.3	44	91.6	1.8	51	20.7
6	47.5	54.3	44	59.6	1.5	40	11.5
7		50.2	92	11.0	1.0	11	2.9
8		20.2	120	35.0	1.0	35	7.0
9		48.5	120	16.4	1.9	9	3.3
C. Diffusion of 0 1 M KCl against 0 1 M $NaNO_3$							
1	53.0		24	3.6	0.8	5	3.6
2		53.5	42	6.1	0.6	10	3.5
3	53 5	51.2	70	13 8	1.4	10	4.7
4	53 5	50.0	70	65.5	4 9	13	22.5

In Series A, where KCl diffused against pure water, the diffused amounts of K^+ and Cl^- are extremely small and, within the experimental error, equivalent to each other.

In Series B and C, where the membrane separated two electrolyte solutions, the amount of K^+ diffused is much higher, and the ratio of diffused $K^+:Cl^-$ is within the range of 4 to 20.

TABLE II.

Progressive Diffusion Experiments with 0.1 N *Solutions.*

Membrane No.	*Co P* of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl
	Before	After		K	Cl	
	mv.		*days*	*mols* × 10^{-6}		
A. KCl against $NaNO_3$						
1	51.5		3	1.6	<1	—
			6	3.3	<1	—
		52.2	12	7.2	1.7	6.6
2	51.0		3	4.6	<1	—
			6	10.8	1.7	6
		37.0	12	24.2	7.4	3
3	52.4		2	14.2	1.8	7.5
			4	31.0	3.2	9.7
			8	70.0	8.0	8.8
		48.8	12	94	13.2	7.0
4	53.7		2	6.6	1.2	5.5
			4	15.0	1.2	12.5
			8	33.0	2.6	12.7
		52.7	12	50.0	5.1	10.0
5	52.7		2	11.6	2.4	5.0
			4	23.4	5.2	4.5
			8	59.0	13.9	4.2
		47.4	12	96.0	16.0	6.0
B. KNO_3 against NaCl						
1	51.0		3	14.3	4.3	3.3
			6	32.2	3.2	10
		41.0	12	67.0	9.2	7
2	53.1		2	11.6	1.2	10
			4	25.0	1.5	16.6
			8	56.0	2.6	21.5
		46.5	12	85.0	5.2	16.3
3	50.2		2	28.3	5.6	5
			4	63.5	9.4	6.6
			8	150	30	5
		41.0	12	214	52	4.1

In this series each membrane was used for a number of successive experiments, with progressively increasing diffusion time. Again, the value of the ratio of diffused K:Cl approaches about 10, sometimes remaining almost the same in the successive experiments, sometimes with a tendency to an increase followed by a decrease in the ratio.

TABLE III.

Membrane No.	*Co P* of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl
	Before	After		K	Cl	
	mv.		*hrs.*	*mols* × 10^{-6}		
A. Diffusion of 0.5 M KCl against H_2O						
1	54.6	50.8	20	1.0	0.8	1.2
2	55.0		92	21.5	18	1.2
B. Diffusion of 1.0 M KCl against H_2O						
3	50.8	50.7	48	0.95	0.85	1.1
4		46.3	216	1.13	1.62*	0.1 appr.
C. Diffusion of 0.5 M KCl against 0.5 M $NaNO_3$						
1		42.5	20	8.5	7.0	1.2
2		42.6	92	13.8	7.0	2.0
3		37.2	360	160.0	155.0	1.0
4		45.2	120	31.0	23.5	1.3
5		51.7	120	37.0	40.4	0.9
6	51.8	51.2	120	84.0	62.5	1.3
7	54.6	50.8	120	96.0	58.0	1.7

In the above experiments with 0.5 or 1.0 M solutions instead of 0.1 M the ratio of diffused $K^+:Cl^-$ is decidedly lower, being approximately the same whether the diffusion takes place against water or another electrolyte.

* Analysis uncertain.

TABLE IV.

Diffusion of Anions Only.

Diffusion of 0.1 M KNO_3 against 0.1 M KCl

Membrane No.	*Co P* of membrane		Time of diffusion	Cl^-
	before	*after*	*days*	*mM*
1	51.5	49.7	12	0.0048
2	51.2	51.0	12	0.0068
3	51.2	39.6	12	0.400

Experiments 1 and 2 show the extremely slight amount of Cl ion diffused in a period as long as 12 days. Experiment 3 shows that a much larger amount of Cl ion diffuses when the character of the membrane has been impaired as shown by the relatively large drop in the *Co P* to 39.6 millivolts.

TABLE V.

Diffusion of 0 05 M KNO_3 against 0 25 M NaCl (No P D. between the Separated Solutions).

Membrane No	Co P of membrane		Time of diffusion	Diffused amounts		Ratios	
				K	Cl	K Cl	u_{K^+} v_{Cl^-}
	before	*after*	*hrs*	*mols* × 10^{-6}			
1	52 4	54 3	196	81	60	1 4	7
2	50 3	52 4	196	47	8 0	5 9	30
3		50 6	240	52 2	3 2	16	80
4	49 3	47 8	196	25	9 6	2 6	13
5	52 4	54 3	196	81	60	1 4	7
6	48 6	47 2	196	34 2	22 0	1 6	8
7		46 0	144	14 8	0 5	30	150
8	57 0	41 5	144	5 0	1 5	3 3	16
9	56 5	39 5	144	7 6	1 1	7	35
10	50 3	37 2	196	48	124 0	0 4	2
11	52 0	24 8	144	7 3	2 0	3 7	18
12	51 0	23 5	144	15 7	14 9	1 00	5

Though the concentration fall of chlorine is five times as great as that of potassium, the amount of diffused K^+ is always greater than that of Cl^-, except in experiment No. 10 The last column shows the mobility ratio of K^+ and Cl^-, referred to the same concentration gradient This ratio is always greater than 1. The exceptionally high value in No 7 may be due to an error in the particularly low amount of Cl^- The experiments are arranged according to the *Co P* values after the experiments However, the figures of the last column do not follow the same order It should be noticed that the *Co P* of many membranes became much smaller after the experiment, showing that the character of the membranes are somewhat changed during the diffusion. This may explain the differences in the ratios

TABLE VI.

Diffusion Experiments with "Gun Cotton" Membranes of Lower Co P 0.1 M KNO_3 against 0.1 M NaCl.

Membrane No.	*Co P* of membrane		Time	Diffused		$K^+:Cl^-$
	Before	After		K^+	Cl^-	
	mv.		*days*	*mols* × 10^{-6}		
1	18.5	26.5	8	7.3	7.1	1.0
2	30.7	22.5	8	87.0	46.9	1.9
3	23.3	23.0	8	9.3	8.3	1.1
4	26.5	26.1	8	35.0	19.4	1.8
5	29.0	22.5	8	10.0	6.2	1.6
6	28.7	23.1	8	64.0	32.8	2.0
The same with "parlodion" membranes						
7	20.0	21.5	8	1.94	2.3	1
8		16.6	26	2.6	2.6	1
9		19.1	26	13.5	8.4	1.6
10		24.3	26	15.8	9.0	1.7
11		23.8	26	23.3	14.0	1.7

This table shows, that in membranes characterized by a low *Co P* (probably due to a larger pore size), the K:Cl ratio approaches 1 and that the specific effect of the membrane on the mobilities of cations and anions disappears.

Discussion of the Diffusion Experiments.

It can be seen from the above tables that the diffusion of an electrolyte (KCl in Table I, A) is slower across the membrane into pure H_2O than into another electrolyte solution. Though the membranes are not uniform enough as to thickness and other properties to permi an exact quantitative comparison of experiments with differen membranes, still it can be seen from Table I, that on the average the amount of K^+ diffusing per day is much less in A than in B or C. This comparison indeed is not very reliable on account of differences in thickness and other properties of individual membranes. However, what is certain is the fact that the amount of diffusing Cl^- corresponds to the amount of K^+ in the diffusion against water (A), whereas it constitutes but a small fraction of the K^+ in the diffusion against another electrolyte (C). In Table I, A, the amounts are so extremely small, that an exact agreement of the analyses cannot be expected. The error of the methods in these very small ranges tends always to yield high values for Cl^-. In any case the order of magnitude for the K/Cl ratio is 1:1, whereas this ratio in B and C is on the average 10:1, varying from 7:1 to 50:1. Only in two experiments in which particularly small amounts had to be analysed the ratio came down to 5:1. Here the error of the method may depress the value, and even if it does not, the ratio 1:1 is far from being reached.

Special attention should be drawn to the experiments shown in Table II, A. Here every membrane was used in a series of successive diffusion experiments with the same combination of electrolytes (KCl against $NaNO_3$) lasting for progressively increasing periods. First it can be seen that Membrane 2, for example, in which the *Co P* dropped in the course of the experiments from 51 to 37.0 millivolts, gives relatively low ratios for the diffusion of K and Cl, dropping down to 3, while the membranes with a higher and more stable *Co P* give ratios of about 7 to 12. In general, within the limits of error, this ratio is approximately the same, though not quite exactly so. It seems that these irregularities are greater than the limits of error in the analyses, and that the regularity and reproducibility of these experiments is not perfect.

The same remarks hold for similar serial experiments with 0.1 M

KNO_3 against NaCl in Table II, B. In general, the amount of potassium diffusing seems to follow a straight line plotted against the time, whereas the chlorine diffusing shows decidedly less regularity. This may be partially due to the greater difficulty in the quantitative determination of these minute amounts of chlorine, but we believe this is not the sole factor.

All of the above experiments were carried out with 0.1 M solutions. In higher concentrations this effect becomes less and less pronounced. So in Table III, with 0.5 M solution of KCl, the amounts of K^+ and Cl^- diffusing are approximately equivalent to each other, both when the diffusion takes place against H_2O and against a 0.5 M solution of $NaNO_3$. It may be recalled that in such high concentrations the P.D. between two KCl solutions of different concentration vanishes (12, *i*), to which problem we will refer in a later communication.

The experiments shown in Table IV in which the cation on both sides of the membrane is the same, but the anions are different, demonstrate that the anions diffuse with extreme slowness, even though the possibility of exchange is present. Membrane 3 of this series, the only one through which a much larger amount of Cl^- diffused, shows at the same time a considerable drop in its *Co P* during the experiments, while Membranes 1 and 2 maintained their original properties as evinced by only small change in their *Co P* values.

A special discussion is required for the experiments shown in Table V. Here an 0.05 M KNO_3 solution was diffusing against five times as concentrated a solution of NaCl. Thus the gradient of the Cl^- concentration across the membrane was five times as great as that of the K^+ ions. None the less, the amount of diffused K^+ is higher than that of Cl^-. If we assume that the velocity is proportional to the driving force, the specific velocities of K^+ and Cl^- can be calculated, in relative terms, by dividing the diffused amount of Cl^- by five. Thus we obtain the ratio of mobility of Cl^- and K^+ shown in the last column of Table V. The reason for carrying out this kind of experiment was the following. In the other experiments with equal concentrations of KNO_3 and NaCl, a potential difference is established. Any solution of a K salt shows a P.D. against any solution of an Na salt in equal concentration across the membrane, which in the best membranes reaches about 50 millivolts (12, *d*). Now, the driving force in the movement of a single species of ion through the membrane is the algebraic sum of the driving force resulting from the concentration difference of this ion on the two sides of the membrane and of the force of the electric field. It is not only the concentration fall which determines the rate of diffusion. Since the side with the Na solution is positive, there is, beside the osmotic force, an additional force, which is directed for positive ions from the Na solution towards the K solution and for negative ions in the opposite direction. Therefore, when the concentration fall for K ion from left to right is the same as the concentration fall for Cl ions from right to left, the total driving force is different for K and for Cl ions. The K ions are retarded, the Cl ions are accelerated by the electric forces. The specific mobility of an ion is its observed speed of movement divided by the driving force. But since the driving force is complicated, the calculation is difficult. The ratio of observed diffusion for K^+ and Cl^- does not represent the ratio of the specific mobilities of these ions. Now, by a suitable arrangement in the concentrations of the K and Na salts, the conditions can be made such that practically no P.D. arises between the two solutions. This is the case when the concentration of the Na salt is about five to ten times as great as that of the K salt. The exact ratio necessary to bring about the complete abolition of the P.D. depends on the individual P.D. of a K-Na chain with the particular membrane. But for a ratio of concentrations 1:5 the P.D. is in any case so low, that the electric force is negligible in comparison with the osmotic force. In fact, the P.D. in the arrangement of the experiments of this series, Table V, was measured in several cases and found never in excess of a few (0.5 to 2.0) millivolts, whereas the same membranes, when interposed between a NaCl and a KNO_3 solution in *equal* concentrations, showed a P.D. of 40 to 50 millivolts according to their individual properties and in agreement with the earlier findings with KCl-NaCl chains (12, *d*). Therefore, the driving force causing the movement of the Cl^- ions may be set simply five times as great as the driving force for K^+. It is probable that the figure five is not quite correct, because neither osmotic pressure nor activity is exactly proportional to the concentration. However, these deviations are certainly within the ranges of the other errors in these experiments.

In this way the "specific mobility" of K^+ and Cl^-, or at least the ratio of the t is calculated in the last column of Table V. It may be emphasized that these ratios hold only for the conditions of this experiment, since the specific mobility of an ion within the membrane depends greatly on the conditions, especially on the concentration, to a much greater extent than it does in a free aqueous solution.

SUMMARY.

The theoretical aspects of the problem of sieve-like membranes are developed.

The method of preparing the dried collodion membrane is described, and the method of defining the property of a particular membrane is given. It consists of the measurement of the *Co P*, that is the P.D. between an 0.1 and an 0.01 M KCl solution separated by the membrane. *Co P* is in the best dried membranes 50 to 53 millvolts, the theoretically possible maximum value being 55 millivolts. Diffusion experiments have been carried out with several arrangements, one of which is, for example, the diffusion of 0.1 M KNO_3 against 0.1 M NaCl across the membrane. The amount of K^+ diffusing after a certain period was in membranes with a sufficiently high *Co P* (about 50 millivolts or more) on the average ten times as much as the amount of diffused Cl^-. In membranes with a lower *Co P* the ratio was much smaller, down almost to the proportion of 1:1 which holds for the mobility of these two ions in a free aqueous solution. When higher concentrations were used, *e.g.* 0.5 M solution, the difference of the rate of diffusion for K^+ and Cl^- was much smaller even in the best membranes, corresponding to the fact that the P.D. of two KCl solutions whose concentrations are 10:1 is much smaller in higher ranges of concentration than in lower ones.

These observations are confirmed by experiments arranged in other ways.

It has been shown that, in general, the diffusion of an anion is much slower than the one of a cation across the dried collodion membrane. The ratio of the two diffusion coefficients would be expected to be calculable in connection with the potential difference of such a membrane when interposed between these solutions. The next problem is to show in how far this can be confirmed quantitatively.

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    - (*c*) Fujita, A., *Biochem. Z.*, 1925, clix, 370.
    - (*d*) Michaelis, L., and Fujita, A., *Biochem. Z.*, 1925, clxi, 47.
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PHOSPHATE ION AS A PROMOTER CATALYST OF RESPIRATION.

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I.

In other papers (Lyon, 1923–24, 1927) results have been reported which appeared to show that neutral solutions of sodium or potassium phosphate serve to catalyze the enzymatic production of CO_2 by plant tissues. The oxidising enzymes involved were those of *Elodea canadensis*, wheat seedlings, and potato tubers, the latter studied only in aqueous extracts. We shall now present additional proof of this promoter action through more careful analyses to determine the active component of mixtures of mono- and disodium phosphates.

The molar concentrations of the solutions which gave optimum results were somewhat high for the usual types of catalysis. The concentration most used was approximately 0.1 M, by which is meant a solution obtained by mixing 0.1 M monosodium phosphate with 0.1 M disodium phosphate. The complex nature of the components of such a solution suggested that some single element among them was the active, or at least the controlling factor of the catalysis. Since the ionization of even this concentration of the sodium phosphates is presumably complete, we are concerned primarily with the nature of the ionization products of phosphoric acid. An excellent statement of the conditions of equilibrium between H_2PO', HPO_4'', and PO_4''' is given by Holt, La Mer, and Chown (1925[1]), from which it is apparent that for H_3PO_4, K_1 is very large, K_2 is smaller, and K_3, which determines the relative amounts of PO_4''', is very small. These authors have also calculated the concentrations of PO_4''' in relation to pH over a wide range and have introduced the expression $p[PO_4''']$ which may be used to express the concentration of this ion just as $p[H^+]$ is used for the hydrogen ion.

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[1] Holt, La Mer, and Chown (1925) pp. 518 to 522.

The relative concentration of PO_4''' is so low that it must be admitted that it is no higher than that of well known catalysts such as the H^+ ion. At pH 7.0 only 1/500,000th of the total P present is present in this ion. On the alkaline side of neutrality the relative concentration of PO_4''' increases rapidly and on the acid side it falls off. A plot of tables of Holt, La Mer, and Chown (1925[2]) shows that the graph of $p[PO_4''']$ (we prefer to use the form pPO_4) against pH is not a straight line but a curve between pH 6 and 9, which correspond to pPO_4 7.44 and 3.4, respectively. Thus pPO_4 will vary with both pH and the molar concentration of the acid or its sodium salts.

Similar statements could be worked out for the other ions of the solutions but it is this ion which proves to be related to catalysis through mathematically simple and exact rules.

As we shall show later, the concentration curve for the rate of CO_2 production by *Elodea canadensis* (Lyon[3]) in different concentrations of neutral phosphate solutions after an exposure of 1 hour does not afford the best data by which to test the conception of catalysis by the PO_4''' ion. At the two extreme concentrations other factors intervene to mask the real effect. When the concentration is low the element of rate of penetration of the phosphate into the living cells limits the observed effect at the end of 1 hour. At the higher concentration there is opportunity for a deleterious effect through either the osmotic effects or some other result of the presence of such a high concentration of salts. The intermediate data are too few to use.

Accordingly, we have performed the experiments necessary to provide data for the effect of change of pPO_4 through change of pH on the alkaline side of neutrality. Similar experiments were attempted for the acid side, but the presence of carbonates or bicarbonates gave rise to such an increase in the liberation of CO_2 that such readings are not comparable with those at a higher pH. In Fig. 1 are shown individual time curves of experiments on the alkaline side of neutrality. The significant values for our purpose are the levels at which each curve flattens out. These experiments were performed according to

[2] Holt, La Mer, and Chown (1925), p. 521.

[3] Lyon (1923–24), p. 302.

the technique described in previous papers, the main apparatus being a suitable form of the Osterhout respirometer (Osterhout, 1918–19, 1919–20). Hence, the preliminary rise in most of these curves is

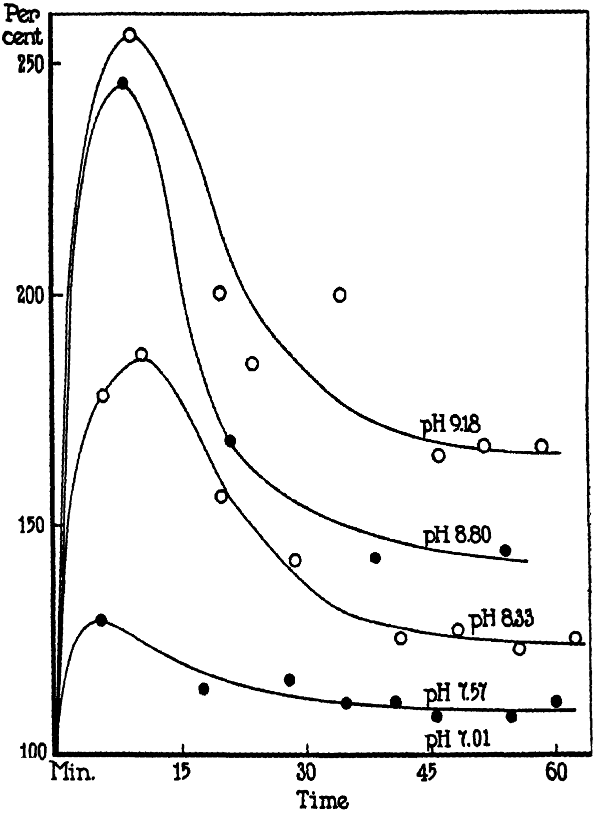

FIG. 1. The effect of alkaline phosphate solutions on the production of CO_2 by *Elodea canadensis*. Each curve represents a typical experiment in which the normal (taken as 100 per cent) is obtained with an 0.106 M neutral phosphate solution and followed by the application of phosphate mixtures of the same molar concentration but with the pH as indicated for each curve.

probably not due to a serious error such as the introduction of atmospheric CO_2 at the time of application of the alkaline solution. It is more likely that we have to do with a temporary change in equilibrium within the system though there may possibly be a real temporary increase in rate of CO_2 production above that at which a level is attained.

In Fig. 2 are shown the mean values (of all experiments) of the rate of CO_2 production at the end of 1 hour, plotted against pPO_4, the latter calculated from the tables of Holt, La Mer, and Chown, and against pOH. The solid line shows the regularity of the relationship between the PO_4''' ion and the rate of production of CO_2. From the

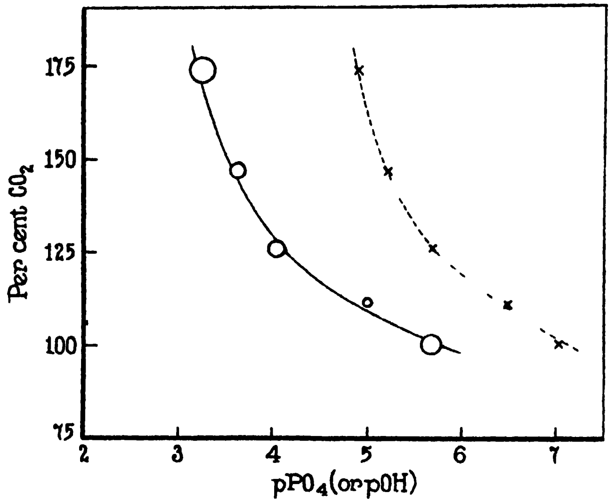

FIG. 2. The mean rates of production of CO_2 by *Elodea canadensis* in 0.106 M alkaline phosphate solutions of different pH in relation to pPO_4 (solid line) and to pOH (broken line), after 1 hour.

nature of the relationship between pPO_4 and pH it is to be expected that the relationship to pOH (broken line) should also be regular. We are attempting to show that the principal effect is that of the PO_4''' ion rather than the OH^- or H^+ ion.

The curve for the relationship to the PO_4''' ion resembles that of a hyperbola with the general equation (for these coordinates)

$$(CO_2 - a)\,(pPO_4 - b) = K,$$

where a and b represent the fact that the asymptotes of this hyperbola may not be $CO_2 = 0$ and $pPO_4 = 0$ but $CO_2 = a$ and $pPO_4 = b$. The mathematical solution of this equation for the five measured points on the curve was accomplished by a reliable method that depends upon the general method of least squares. The equation which results is

$$(CO_2 - 68.475)\ (pPO_4 - 2.13) = 114.43.$$

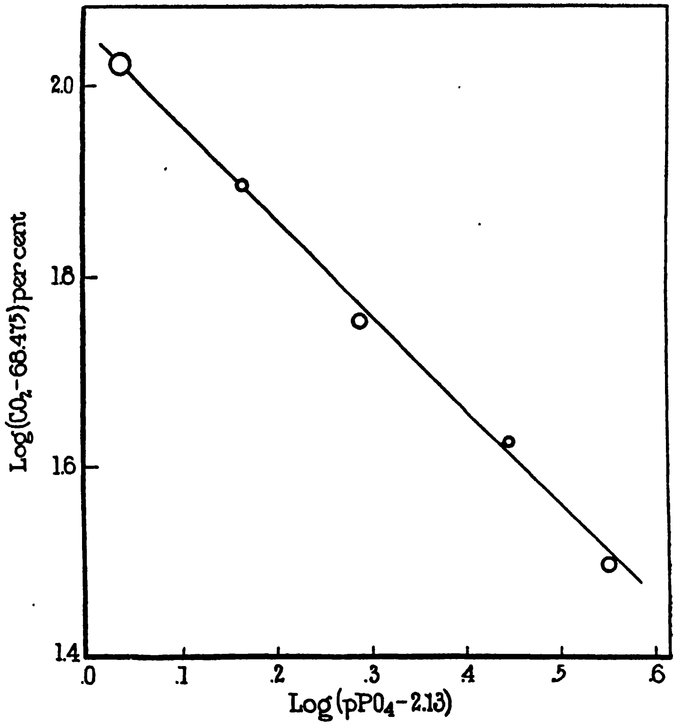

FIG. 3. The rates of production of CO_2 by *Elodea canadensis* in 0.106 M alkaline phosphate solutions of different pH in relation to pPO_4. The ordinates are logarithms of per cent $CO_2 - 68.475$ while the abscissæ are logarithms of $pPO_4 - 2.13$. The straight line is the plot of values calculated from the equation in the text.

The closeness of fit to the data may be seen in Fig. 3 in which the logarithm of the per cent CO_2 is plotted against the logarithm of pPO_4. If the hyperbolic relationship holds the points should lie in a straight line in this type of plot. The straight line we have drawn represents the calculated equation while the points indicate the locations of the measurements.

The meanings of the constants *a* and *b* are interpreted as follows: Under the conditions by which the relationship was derived (*viz.* with the pH also increased) the one asymptote locates the point beyond which further increase in concentration of PO_4''' ions fails to give an increase in rate of CO_2 production; the other asymptote denotes the fact that the enzyme may function apart from PO_4''' ions to the extent of 68.475 per cent, where 100 per cent is the rate measured in neutral phosphate solution (0.1 M). Not much emphasis can be attached to these constants, however, since they were not obtained by changing only one variable—an impossible step in this work.

We may now observe the relation of this equation to the points on the concentration curve for *Elodea* (Lyon, 1923–24, Fig. 2). The horizontal form of the curve at molecular concentrations approaching the threshold of plasmolysis could not be expected to check with an equation derived from measurements at optimum salt concentrations. Some limiting factor may also prevent a close adherence to a hyperbolic relationship.

Likewise, the lowest concentrations might not exhibit the same relation. Here it is found that if the increase in molar concentration be thought of as a slow increase in concentration of PO_4''' ions (as is the case), the slope of the curve for CO_2 production is greater than that of Fig. 2 and is sensibly uniform over the first third of the graph. This linear, or very nearly linear relationship, which denotes direct proportionality, is typical of catalytic processes over the range of low concentrations and does not conflict with the relationship expressed by our derived equation. At the midrange of concentration, however, we should expect some conformity and such was found to be the case. The total difference between the K's computed for the two known midpoints is only 1.5 per cent of the mean K.

This is as far as we can go in the analysis of our own data which seem to point to the PO_4''' *ion as the effective* catalyst of oxidising enzymes. It would be very desirable to obtain similar concentration curves for other plant material, by experiments involving change of concentration of the phosphate ion by changing first the pH and then the molar concentration. We plan to conduct such studies at a later date.

The results of Bode (1926), who sought to measure the dependence of respiration upon hydrogen ion concentration, show a qualitative agreement with our results in so far as his data may be converted into data for pPO_4. His use of calcium and magnesium phosphates to regulate the pH introduces new variables affecting the exact concentration of t[illegible]sphate ion and involving the influence of the metal. In general [illegible]ever, the presence of higher concentrations of phosphate ion correlates with a higher rate of respiration as measured by the absorption of oxygen.

II.

A further confirmation of the promoter action of the phosphate ion is afforded by the results of analyses of some existing records in the literature of other enzyme actions. Appropriate treatment of such records demonstrates the same hyperbolic relationship.

The widespread practice of using phosphate buffers for controlling the pH of enzyme reactions has led to the statement of some records in a form which allows the transposition of either molecular concentration or change of pH into data for pPO_4. Unfortunately it is common practice to report but a few "typical" series of measurements. Mean values of two measurements were found for some cases and of course these as well as data covering both change of pH and change of molar concentration at constant pH in the same type of experiments carry more weight.[4]

The method of procedure in the conversion of data into terms of pPO_4 will be described for only the first set of readings to be dealt with—those given by Smirnoff (1925) in a study of the effect of neutral salts on peroxidase. The enzyme was obtained from ground wheat seeds. The substrate was pyrogallol. The criterion of enzyme action was the amount of purpurogallin formed, estimated by titration with $KMnO_4$. The data for the effect of the phosphate solution of concentration N/80 were given for only one set of experiments, in the following tabular form.

pH	3.5	4.0	4.98	6.5	7.1	7.5	7.86	8.3
Activity of enzyme, per cent	1.9	2.84	55.79	100.0	135.0	148.16	150.53	175.07
pH	8.7		9.3	9.5				
Activity, per cent	183.64		173.86	139.68				

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[4] The measurements, however, are all recorded in terms of amounts of product after equal periods of time. That this is not always the proper measure of enzyme action was shown in the recalculation by Northrop (1924-25) of the results of Morgulis (1921) in a study of the kinetics of catalase action on peroxide.

From these data it is obvious that below pH 4.98 there is a powerful inhibition of enzyme action and above pH 8.7 there is a depressive action. These effects are undoubtedly due to a specific effect of the pH value and we can only use the intermediate data. The necessary data for a logarithmic plot in terms of pPO_4 is given in the following table. The pPO_4 was obtained by interpolation from a plot of the table of pPO_4 for various pH values.

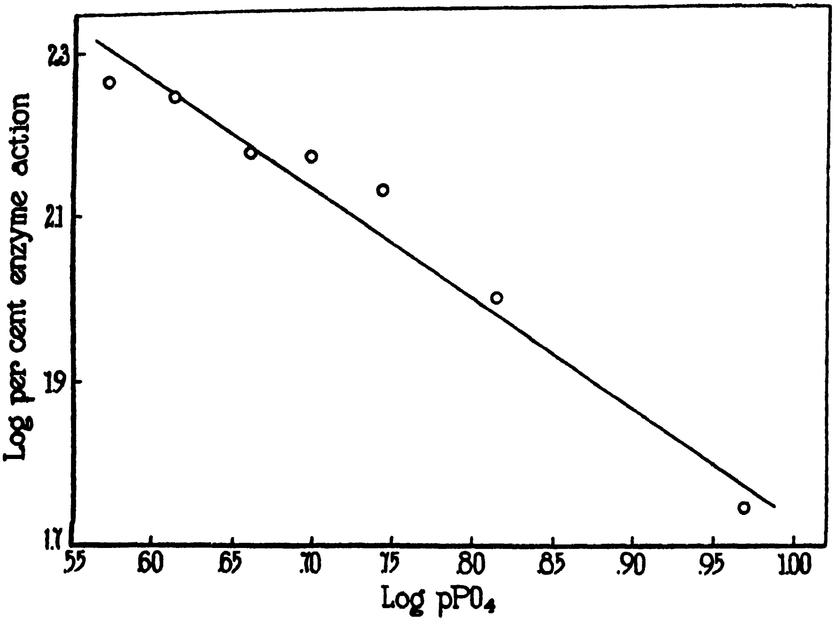

FIG. 4. The logarithmic plot of the relation of the concentration of PO_4''' ions to the percentage rate of oxidation of pyrogallol to purpurogallin by peroxidase. The straight line is the plot of the equation

$$(\text{Activity of enzyme})\,(pPO_4)^{1.34} = K.$$

The indicated points represent a single set of readings as reported by Smirnoff (1925).

pH	pPO_4	Log pPO_4	Enzyme activity in per cent	Log enzyme activity
4.98	9.43	0.9745	55.79	1.7465
6.5	6.51	0.8136	100.0	2.0000
7.1	5 53	0.7427	135.0	2.1303
7.5	5.00	0.6990	148.16	2.1707
7 86	4.58	0.6609	150.53	2.1775
8.3	4.10	0.6128	175.07	2.2430
8.7	3 72	0.5705	183.64	2.2640

Fig. 4 shows the plot of these values. The points lie as near to a straight line as could be expected for a single set of readings. The slope of this line is not −1, however, but −1.34. Therefore the equation is

$$(\text{Activity of enzyme})\ (\text{pPO}_4)^{1.34} = K.$$

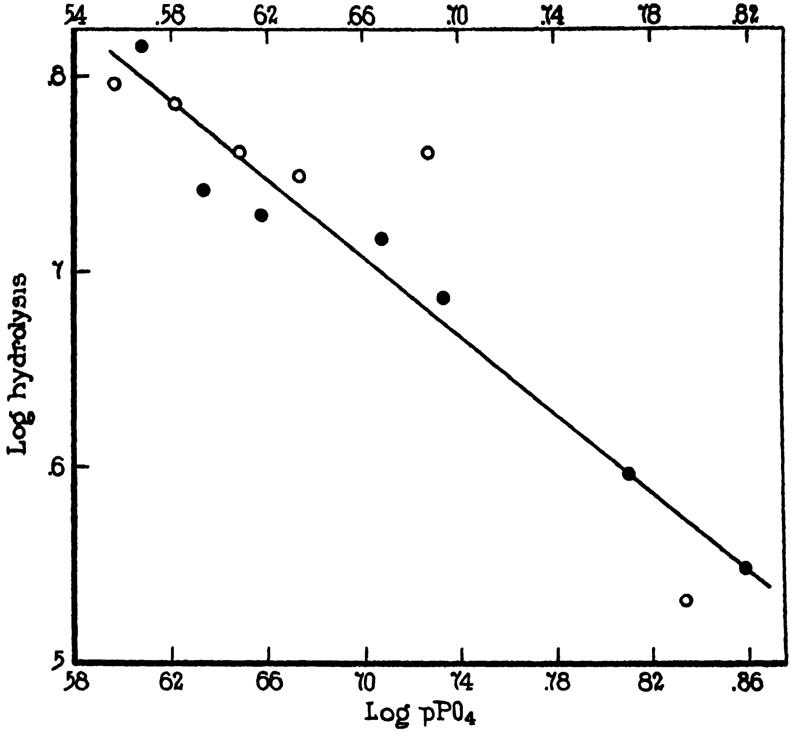

Fig. 5. Logarithmic plot of the relation of concentration of PO₄‴ ions to the rate of hydrolysis of ethyl butyrate by pancreatic lipase in glycerin extracts of the pig pancreas. Data are taken from the work of Platt and Dawson (1925) who varied pPO₄ by change of pH. The straight line is fitted to the points (●) obtained from the readings for an experiment involving the use of low concentration of phosphate salts. The abscissæ are those from the scale at the bottom of the figure and the equation for the line is

$$(\text{Hydrolysis})\ (\text{pPO}_4)^{1.01} = K.$$

The other points (○) are referred to the scale of abscissæ at the top of the figure, this scale differing from the lower one only by a displacement to the right by four units. These points were taken from a second experiment which differed only in the use of five times as much phosphate salts in the buffer solutions.

Smirnoff did not study in sufficient detail the effect of the concentration of phosphate solutions to warrant a similar test of the phosphate ion relations at constant pH. The method of calculation of pPO_4 under those conditions is fundamentally the same and will appear in connection with lipase analyses.

For another of the oxidising enzymes—laccase from alfalfa—we have found a single set of four readings for change of pH as recorded by Bunzel (1915). Its action was tested only on hydroquinone. The "unnaturalness" of the conditions is attested by the fact that there is no absorption of oxygen in neutral solutions. Therefore no conclusions can be reached as to the relation of laccase to the phosphate ion in living cells in which the substrate is quite different and the enzyme is effective at the pH of protoplasm. Actually, the graph on the logarithmic plot was a straight line with slope -7.46 for three of the four readings, the fourth one being nearest the point of no oxidation.

The most complete data to be found for enzyme action in relation to phosphate are those for pancreatic lipase. In all, the results of five independent sets of experiments have been found to be available for analysis. The most recent workers (Platt and Dawson, 1925) recognized a specific function of the phosphate buffers which were also used by the previous workers. Their technique was likewise more refined and their data cover both change of pH and change of concentration. Hence, their results carry more weight and are considered first.

Platt and Dawson estimated the action of pancreatic lipase of the pig by titrating the butyric acid released through the hydrolysis of ethyl butyrate. They were concerned partly with the optimum pH and found it to be about 7.0 for phosphate buffers and purified lipase. By using glycerin extracts of the pancreas they were able to carry the pH as high as 8.0 with steadily increasing hydrolysis. They consider that a protection of the enzyme is afforded by some constituent of the glycerin extract. These experiments (Nos. 5 and 6) are therefore the ones from which we have obtained data for a wide change of pH with constant concentration of phosphate salt. They are shown in Fig. 5 from which it is evident that their Experiment 5, in which only 5 cc. of phosphate solution was used, gives an unbroken straight line through a wide range of pPO_4. Experiment 6 involved the use of 25 cc. of phosphate solution and the points (shown by ○) are somewhat irregularly distributed. In both experiments the points represent single readings and additional experiments would probably remove the irregularities. The general trend of the points is not far from that for the lower concentration of salt. the equation for which is

$$(\text{Hydrolysis})\ (pPO_4)^{1.01} = K.$$

After a specific effect of phosphate was observed the point was carefully studied by using different molar concentrations of the buffer salts (*no hydrolysis could be observed in the absence of phosphate*). In the first of these studies (their Experiment 9) low concentrations were employed at pPO_4 4.9 (pH 7.6). The results show that for the range 0.005 to 0.05 M there is a hyperbolic relationship between the

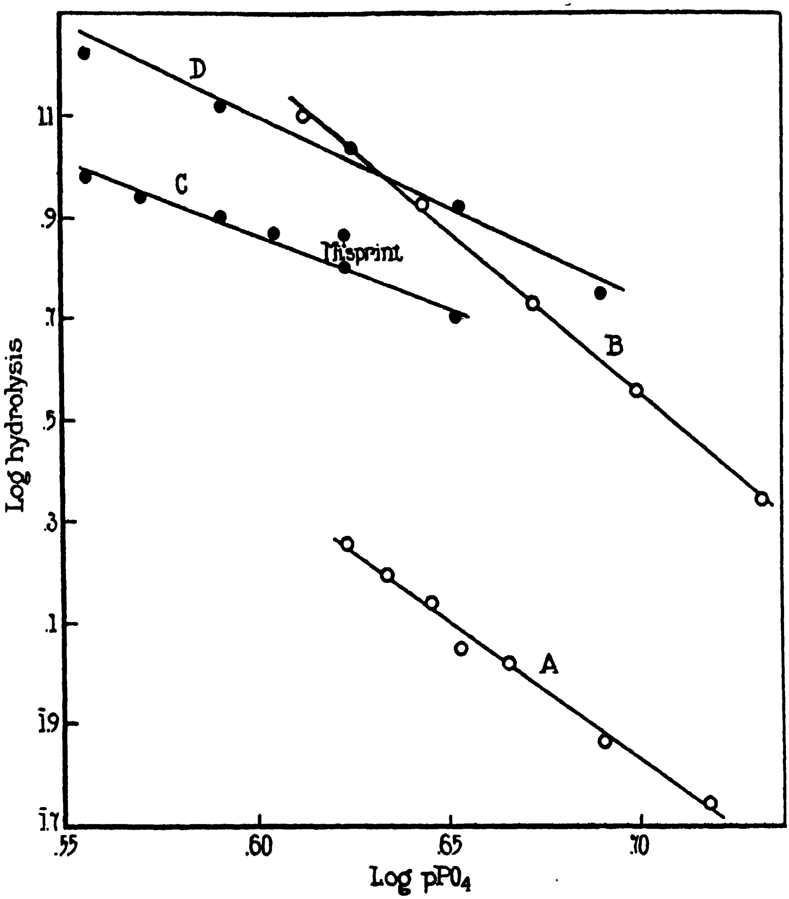

FIG. 6. Logarithmic plot of the relation of pPO$_4$ to the rate of hydrolysis of ethyl butyrate by pancreatic lipase. Data are taken from the work of Platt and Dawson (1925) in which the pPO$_4$ was varied by the use of varying concentrations of phosphate salts at constant pH. Each point represents the mean value of two readings. The equations for the lines, together with the pH used for each, are as follows:

Curve A (Hydrolysis) $(\text{pPO}_4)^{5.64} = K$ pH = 7.6
" B (Hydrolysis) $(\text{pPO}_4)^{6.32} = K$ pH = 7.2
" C (Hydrolysis) $(\text{pPO}_4)^{2.9} = K$ pH = 7.6
" D (Hydrolysis) $(\text{pPO}_4)^{3.59} = K$ pH = 7.6

The misprint indicated in connection with Curve C was obviously a mistake in one figure of the number given in the original paper.

amount of hydrolysis and the pPO_4. The logarithmic plot is given in Fig. 6, Curve *A*, which indicates the relation

$$(\text{Hydrolysis})\ (pPO_4)^{5.64} = K.$$

The method of obtaining the pPO_4 for the various concentrations is the same as that used in similar conversions for pH and was done by the use of the table of factors given by Clark (1922[5]) for this purpose. Thus the pPO_4 at 0.01 M (all calculations were corrected to this concentration) is 4.9 while that at 0.03 M is 3 × 4.9 or 0.3 × 3.9 which is pPO_4 4.42.

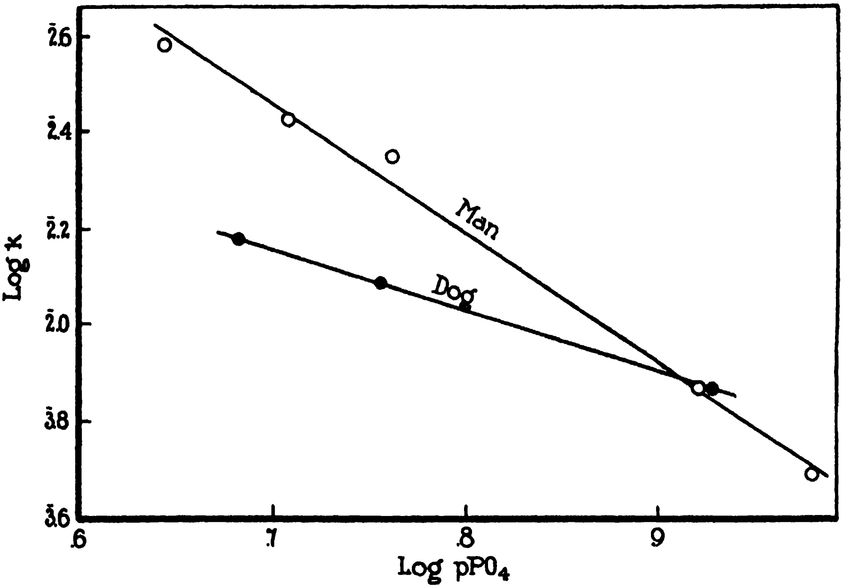

FIG. 7. Logarithmic plot of the relation of pPO_4 to the action of pancreatic lipase on tributyrin. Data are taken from the work of Rona and Pavlović (1922). The pPO_4 was varied by using buffer solutions of pH 4.94 to 8.01. The equations for the lines are

for lipase from dog (●) $- K\ (pPO_4)^{1.26} = K$
" " " " (○) $- K\ (pPO_4)^{2.7} = K$

Each set of points is taken from a single experiment.

In Fig. 6 there are also shown the results of the remainder of the tests with change of concentration at constant pH. For the experiment shown by Curve *B* the pH was 7.2. This is nearer the optimum than was the case for Curves *C* and *D* in which the pH was 7.6. Platt and Dawson comment on the difference in form between these two types of curves as plotted using molar concentration, and say that the farther the pH is above the optimum, the more pronounced the curvature. This is somewhat noticeable in Curves *C* and *D* when the data are converted to a logarithmic plot. However the conditions in the experiments were

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[5] Clark (1922), p. 456.

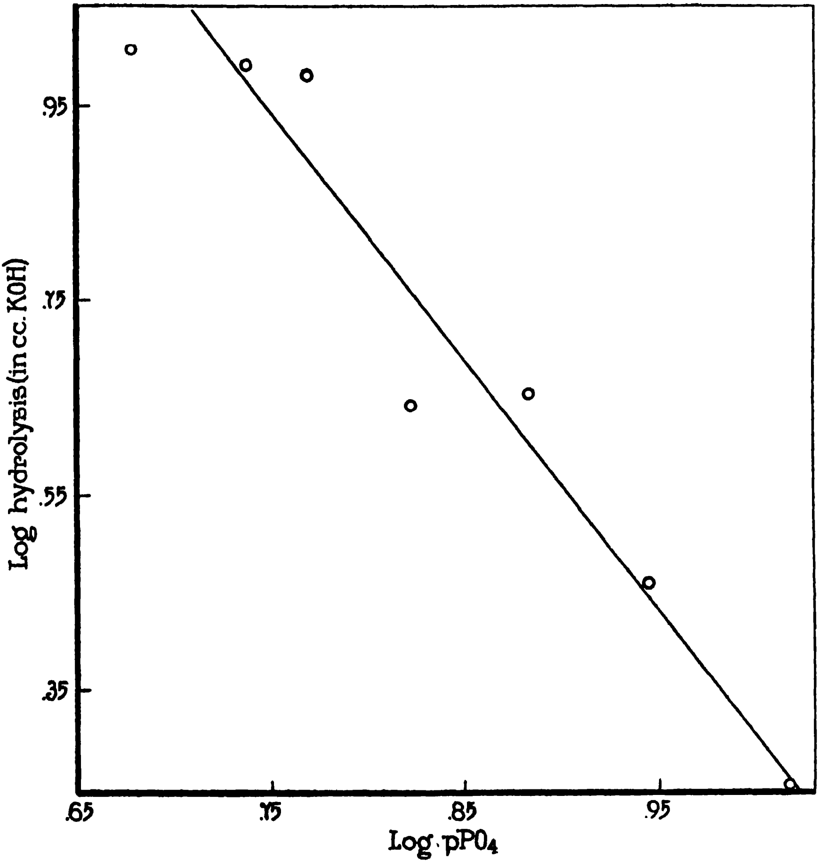

FIG. 8. Logarithmic plot of the relation of pPO_4 to the hydrolysis of olive oil by pancreatic lipase of the pig. Data are taken from a single experiment by Umeda (1915). The pPO_4 was varied by the use of buffer solutions of pH 4.494 to 7.731. The equation for the line as drawn is

$$(\text{Activity of enzyme})\ (pPO_4)^{2.54} = K.$$

essentially the same as for those shown by Curve *A*. (This was also our justification for suggesting the indicated misprint in the data used for Curve *C*; see legend).

The equations for the four experiments in which the pPO_4 was altered by change of molar concentration are given in the legend of Fig. 6. They differ from the equation for the same relationship derived by change of pH only in the slope of the line, which is also indicated by the exponent of pPO_4 in the equation. This difference is directly attributable to the attendant differences in molar concentration and hydrogen ion concentration. The straight lines on the logarithmic plots attest the hyperbolic relationship between pPO_4 and activity of the lipase, *whether the concentration of the PO_4''' ion be increased by decrease of pH or by increase of total salt concentration.* The slope merely measures the sensitivity of the given sample of enzyme to the change in concentration of the PO_4''' ion under the experimental conditions imposed by other factors.

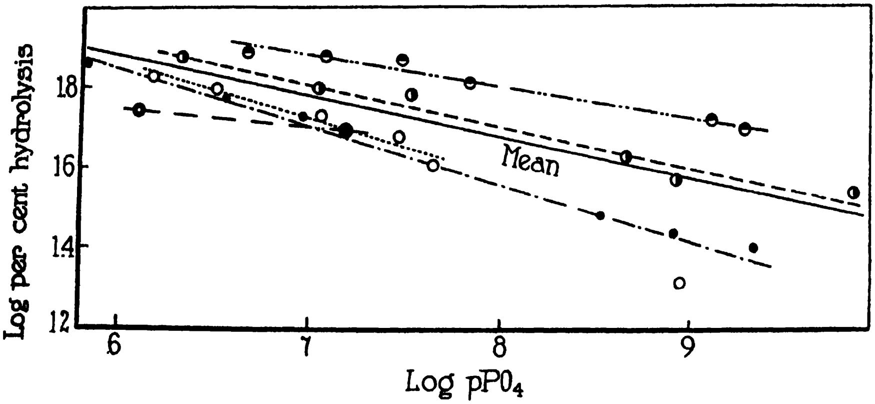

FIG. 9. A composite logarithmic plot of the relation of pPO_4 to the percentage hydrolysis of tributyrin by the pancreatic lipase from beef pancreas. Data are taken from five single experiments by Rona and Bien (1914), each of which is represented by a broken line. The equation for the mean line as drawn (solid line) is

$$(\text{Hydrolysis})\ (pPO_4)^{1.08} = K.$$

The pPO_4 was varied by the use of buffer solutions of pH 4.87 to 8.58.

Platt and Dawson also noticed that both *a*- and *b*- sodium glycerophosphate promoted the action of lipase to the same degree. They state their opinion that this indicates an effect by the phosphate ion. The present analysis confirms this observation.

Another recent measurement of the action of pancreatic lipases (purified) on tributyrin is reported by Rona and Pavlović (1922). The available data are very meager but the uniformity of the results is evident from the form of the plots in Fig. 7. Only three measurements are available for the lipase from dog pancreas and there are but five for that from the human pancreas. Each set of points, however, lies along a straight line, the slope of which differs for the two types of enzymes. The "*k*" used as a measure of the activity of the enzyme was calculated by the authors from a monomolecular equation for the hydrolysis. In each case the pPO_4 was altered by change of pH.

The work of Umeda (1915) furnished a single set of readings of the hydrolysis of olive oil by purified pancreatic lipase of the pig, in relation to pPO$_4$ as calculated from the pH. The lipoclasis was estimated by titration of the acid produced after 20 hours. Fig. 8 shows the same hyperbolic relationship to pPO$_4$. Here the equation is

$$(\text{Activity of enzyme})\ (\text{pPO}_4)^{2.84} = K.$$

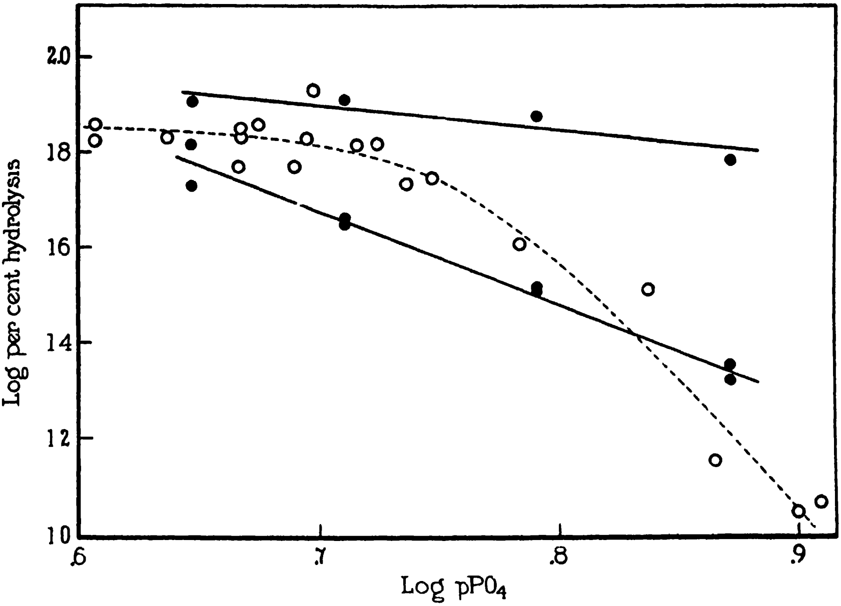

FIG 10 Logarithmic plot of the relation of pPO$_4$ to the percentage hydrolysis of fat by pancreatic lipase of the pig (shown by (●) and solid lines) and by lipase of the duodenal juice (shown by (o) and broken line). Data are taken from single readings by Davidsohn in 1913.

Rona and Bien (1914) used a glycerin extract of lipase from beef pancreas and estimated the hydrolysis of tributyrin by a stalagmometric method. This method is not so accurate as titration. The results are converted into percentage of hydrolysis and plotted against pPO$_4$ as usual in Fig 9. This is a composite of several experiments, each of which is indicated by a broken line. The variation from the average line is due largely to the variation in the activity of the sample of enzyme used Each experiment gave a straight line plot and the mean slope is −1.08. Therefore the equation is the same as those derived from later measurements.

In Fig. 10 are shown the results from a still earlier report (Davidsohn, 1913) of hydrolysis of fat by pancreatic lipase of the pig. This is the only case we have found in which all the points do not lie along a straight line on this type of plot. The two solid straight lines in the figure are drawn in respect to the readings from three experiments with lipase direct from the pancreas, one sample of which was obviously more active than the other two. These data clearly exhibit the same type of relationship as has been demonstrated in the previous analyses.

The other points ($\bar{o}$) on this composite plot represent data from a number of single measurements with lipase contained in the duodenal juice. The curved, broken line roughly indicates the trend of these points as plotted over a wide range of pH and pPO_4. The points at the extremes could be accounted for on the basis of the unfavorable pH of the media. However, the distribution of all these points is not only very irregular but uncertain. This is due to the composite nature of the data, the use of the stalagmometric method of analysis, the variation in the total concentration of phosphate buffer salts, and the inability to correct for all such factors in converting to pPO_4 because of the incompleteness of the statement of experimental procedure. The relationships exhibited by our other analyses of lipase studies and the recent demonstration by Platt and Dawson of an absolute dependence of pancreatic lipase, also of the pig, upon the presence of at least some phosphate, appears to outweigh the doubtful evidence from these older results as regards the lipase obtained in a different medium.

From these analyses of the relation of lipase, peroxidase, and possibly laccase to phosphate solutions, it seems quite certain that the PO_4''' ion acts as a promoter of their activities. The mathematical statement of the relationship is like that for the effect of phosphate on the production of CO_2 by living cells. There is then every reason to believe that the active component of such phosphate solutions is the PO_4''' ion, acting as a promoter catalyst. The very fact that the mathematical expression of the relationship is of the form

$$(\text{Activity of enzyme})\,(pPO_4)^n = K$$

is of itself an additional proof; for the term "pPO_4" is a direct measure of the potential of the PO_4''' ion in a given solution. The inverse proportionality expressed by the equation is really a direct proportion because of the peculiar method of statement of the potential.

In the case of plant respiration the exponent of the pPO_4 term was found to be 1. The corresponding exponent in the case of peroxidase was essentially the same (1.34). Although an exponent of this order was also found for a few cases in the lipase analyses (*cf.* Figs. 5, 7, and 9), the value 3 or 6 was more characteristic of lipase (*cf.* Figs. 6 to 8). The agreement of the numbers of the "oxidase" group of enzymes is significant while the question of sensitivity of lipase (measured by the value of the exponent, *n*) to the phosphate ion is beyond the immediate scope of our problem.

III.

SUMMARY.

The active component of phosphate solutions, in relation to promoter action on oxidising enzymes, is the PO_4''' ion. This is shown by the demonstration of a hyperbolic relationship between per cent production of CO_2 (of *Elodea*) and pPO_4, the measure of the phosphate ion potential. This is consistent with the rate of respiration as affected by changing pPO_4 through change of total phosphate concentration while pH is kept constant. The equation for this relationship is

$$(CO_2 - a)\ (pPO_4 - b)^n = K$$

where a, b, n, and K are constants and $n = 1$.

The same relationship to phosphate ion concentration, expressed by the equation

$$(\text{Activity of enzyme})\ (pPO_4)^n = K,$$

where n and K are constants and n varies from 1 to 6 under different conditions, appears to hold for some other enzyme actions, including those of peroxidase and pancreatic lipase.

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ARSENATE AS A CATALYST OF OXIDATION.

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In connection with another paper describing the catalytic rôle of phosphate in plant respiration (Lyon, 1927) brief mention is made of some experiments in which the effect of arsenate on plant respiration and on oxidation was observed qualitatively. A catalytic effect is found in both cases. The importance of this hitherto unconsidered property of arsenate seems to justify a more detailed report of experiments which have been extended in order to provide more data.

It should be borne in mind that the present discussion of the catalytic effect of arsenate does not detract from the apparently unique rôle of phosphate as a non-toxic promoter catalyst for the respiratory enzymes. The same concentration of arsenate which momentarily increases the rate of production of CO_2 by living tissues soon exercises a toxic effect.

There are several suggestive items of evidence to be found in the literature regarding the effect of arsenate salts. Most of those to which we shall refer have resulted from comparisons of arsenate with phosphate in models or isolated phases of respiration. These are therefore particularly valuable items of evidence.

In the oxidation-reduction system of Haehn and Pülz (1924) a supposed cleavage of water molecules is effected by means of a combination of amino acid and phosphate. At least the presence of these two reagents in aqueous solution results in an oxidation of acetaldehyde and a reduction of methylene blue, at relatively high temperatures. Arsenate was found to be equally effective when substituted for the phosphate.

Meyerhof and Matsuoka (1924) have repeated and extended the observations of Warburg and Yabusoe (1924) on the oxidation of fructose by pure oxygen in a solution of phosphates presumably containing iron salts. They found that arsenates could be used in place of phosphates without destroying the effectiveness of the iron catalysis.

The substitution of arsenate for phosphate in alcoholic fermentation cannot be made to the extent of a complete replacement of phosphate. Some phosphate is essential to the formation of hexose phosphate, an ester of hexose and potassium phosphate. This ester is continually being formed and decomposed by certain enzymes. Harden and Young (1911) did observe that arsenate could replace phosphate in the sense that the latter causes an increase in the production of CO_2 (and alcohol) They attributed this effect to an increased activity of hexose phosphatase, the enzyme which splits the ester

When Harden and Henley (1922) reviewed the work of Witzemann (1920) on the oxidation of glucose by H_2O_2 as affected by phosphate, they were inclined to the view that the buffered solution of phosphates aided the reaction by the effect on the peroxide. They demonstrated a qualitatively similar action by other salts such as carbonates However, their results show a special effect of phosphates and arsenates over and above the buffer action This effect has never been clearly explained

Our first experiments with arsenates consisted in adding them to slowly oxidizing solutions of pyrogallol and to slowly respiring tissues of *Elodea canadensis.*

Whenever a few drops of arsenate solution—either the alkaline ($pH = 9.18$) solution of disodium arsenate or the same solution brought to pH. 7.0 with the same molar concentration of arsenic acid—are added to a relatively large volume of a solution of pyrogallic acid there is a rapid coloration of the solution. This indicates an oxidation of the pyrogallol, the end-products being purpurogallin, carbon dioxide, and water. It is a matter of hours before the usual slow oxidation forms sufficient purpurogallin to give a pale straw color to the solution, but the addition of arsenate provides the same color in not more than 2 minutes. The effect of arsenate is qualitatively the same as that of phosphate (*cf.* Lyon, 1927).

In a typical experiment 5 drops of a 0 085 M solution of disodium arsenate are added to 15 or 20 cc of 1 per cent solution of pyrogallol. For the control there is added the same number of drops of a solution of NaOH which has been diluted until the hydroxyl ion concentration is the same or greater than that of the arsenate solution. The difference in color between the experiment and control increases rapidly during the 1st hour or 2 and is more marked near the surface of the solution The difference is maintained for days and weeks while both solutions become yellowish, then brown, and finally brownish-purple No previous mention of such an effect has been found.

In Fig. 1 are shown six individual time curves of the rate of production of CO_2 by *Elodea canadensis.* These experiments were carried out according to the technique described in a previous paper (Lyon, 1923-24), using a form of the apparatus described by Osterhout (1918–19). The normal rate of production of CO_2 by the untreated tissues is taken as 100 per cent. The subsequent changes in the rate are indicated by a curve smoothed through points each of which represents the rate measured at a certain time after the addition of a sufficient volume of

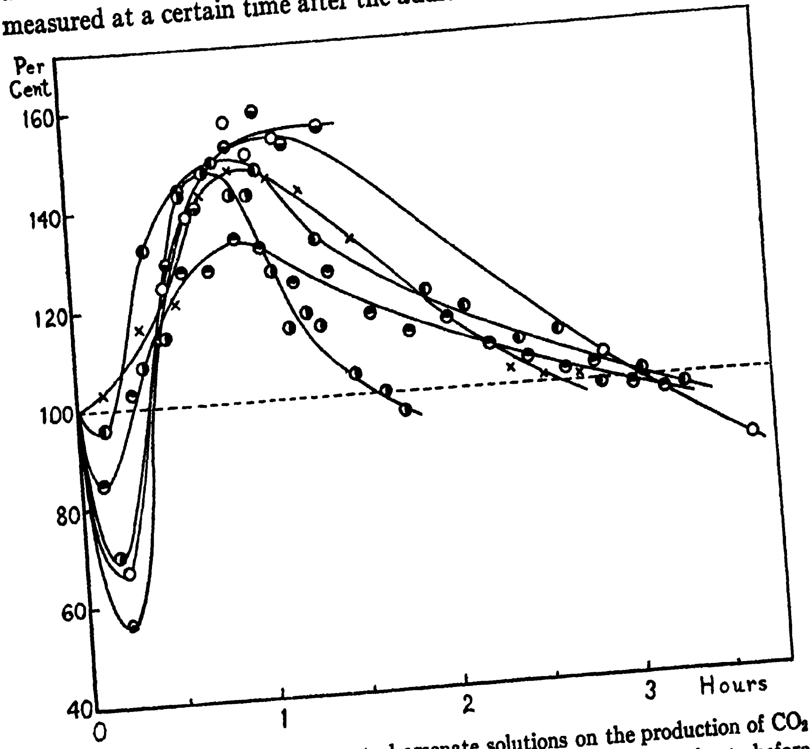

Fig. 1. The effect of 0.1 M neutral arsenate solutions on the production of CO_2 by *Elodea*. Each curve represents a single experiment. The normal rate before the addition of arsenate is taken as 100 per cent.

a concentrated solution of neutral mixtures of disodium arsenate and arsenic acid to give an effective concentration of 0.1 M.

The fall of the first readings below normal is to be regarded as the result of the increased CO_2-absorptive capacity of the diluted solutions of arsenates (*cf.* Fig. 1 in Lyon, 1923–24). The rise of each curve above the 100 per cent level shows the manner in which the rate of production of CO_2 is uniformly increased after about 1 hour. The similarity of this part of each curve with that of the individual curves for the effect of phosphate (*loc. cit.*) is evident. This close parallel may lead us to believe that arsenate also acts as a catalyst to the respiratory enzymes of *Elodea.*

The difference in the effects of arsenate and phosphate is shown by the difference in their time curves after the 1st hour. With arsenate the toxic effect is shown by the gradual decrease in the rate of production of CO_2 until the rate becomes slower than the normal. With phosphate no such toxic effect is observed. The differences to be noted among the several individual experiments are to be attributed to the different rates at which the several lots of plants showed susceptibility to the toxic effect of the arsenate.

Until this apparent catalytic effect of arsenate has been demonstrated in a greater number of cases we may not go far in using the principle in an interpretation of the general action in organisms. We would, however, point out a possible relation of this catalysis to three of the four cases cited to show how phosphate may be replaced by arsenate. In the system used by Haehn and Pülz, in that of Meyerhof and Matsuoka, or in that of Harden and Henley, the substitution of arsenate for phosphate may be possible because each acts as a catalyst in the system. For example, in the case of the oxidation of fructose by pure oxygen in the presence of phosphate or arsenate and of iron, the phosphate or arsenate may act as a promoter catalyst to the slow or potential oxidation of fructose by iron.

To test the principle involved in this hypothesis we have performed, with arsenate, experiments comparable with those reported elsewhere for phosphate (Lyon, 1927). Into each of four tubes were placed 15 cc. of a 1 per cent solution of pyrogallol. Additions of substances selected for an analysis of their effect on the rate of oxidation were made as follows: (1) 4 drops of 0.34 M neutral arsenate mixture; (2) the same plus a small, clean, iron nail; (3) 4 drops of water plus a similar iron nail; (4) 4 drops of water. No. 4 was a control and the rate of oxidation was so slow that no important color change could be observed for hours. The arsenate in 1 induced a visible production of color after not more than 2 minutes. Essentially the same color appeared in 2 above the nail and even less color finally came in 3 above the nail than in 4. But at the surfaces of the iron there appeared strongly colored regions. The region in 2 was much greater in volume than that in 3 but was violet-purple in contrast with the pure purple about the iron in 3.

During the remainder of such an experiment the tubes were frequently stirred in order to avoid gradients in the concentrations of oxygen and oxidation products throughout the solution, particularly in the vicinity of the iron. This procedure caused no change in the apparent color of the oxidation products, but after 2 to 4 hours it was observed that the density of the coloration in No. 2 was much greater than that in either 1 or 3. To determine whether this was anything more than the additive effect of the two catalysts, the color was compared with that of a mixture of equal parts of 1 and 3, care being taken to equalize the dilution effect of the mixture. The result of such comparisons showed that the color in 2 was of a density greater than the purely additive effect would account for. It thus fully supported the hypothesis that arsenate can function as a catalyst to an iron catalysis—the so called "promoter effect."

These experiments have been repeated many times with uniform results, and likewise with pure iron wire to show that the iron in the nail alone catalyzed the oxidation. Attempts to obtain time curves for these experiments have been prevented by inherent difficulties. It is possible to find a suitable color standard for either the coloration produced by the arsenate and iron acting together, or for that of the color of the mixtures of solutions in which each acts alone; but the same standard will not do for both. This is due to an excess of pure purple produced when they act together and which is of itself a demonstration that the arsenate influences the action of the iron as a catalyst. By definition this is a promoter effect and corresponds to the observed effect on the respiratory enzymes of *Elodea* up to the time when it was masked by the toxic effect.

SUMMARY.

Arsenate exerts a catalytic effect on the oxidation of pyrogallol by atmospheric oxygen, on the catalytic oxidation of pyrogallol by metallic iron, and on the presumably enzymatic production of CO_2 by *Elodea canadensis*.

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A THEORETICAL CONSIDERATION OF THE ACTION OF X-RAYS ON THE PROTOZOAN COLPIDIUM COLPODA.

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I. INTRODUCTION.

To show that the variation in reaction among the individuals of a group of apparently similar organisms fits a probability curve is, of course, not an end in itself, but rather a basis of conjecture as to the mechanism which provides the random element without which the theory of probability is meaningless. It is generally assumed that the reactions of various individuals differ because the individuals themselves are different. It is interesting, however, to consider the possibility that in some cases, where the organism is relatively simple, the individuals are essentially similar and the random element is inherent in the agent or in its primary effects. For example, if the agent is radiation, the quite generally accepted theory of absorption in *quanta* provides the necessary random element; and it may be that this is sufficient to account for the variations which we observe in the reaction to the rays of apparently similar simple organisms.

In his recent work on the action of x-rays on *Colpidium colpoda*, Crowther[1] finds that if he plots the number of survivors against the dose of radiation administered the result is a sigmoid curve; and, assuming the animals to be essentially alike, he shows that this is the kind of curve to be expected if the animal dies as a result of a certain number, n, of discreet events or "hits," all equally effective, and if the probability per unit dose of making a hit is constant. He finds, further, that theory agrees quantitatively with experiment when λ_e, the probability per e-unit of dose, is 5.9×10^{-4}, and n (for immediate death) is 49.

To account for this very small value of λ_e, he makes use of an interesting hypothesis, which he advanced some time ago[2] for a similar purpose, that the effect is confined to some very small body or structure inside the animal. In doing so, he introduces certain difficulties, in my opinion. No general objection is raised against this hypothesis, which we may refer to as the "small-body theory." In this particular case, however, it appears to be inconsistent with the phenomenon which it was devised to explain, unless it is supplemented by certain rather novel assumptions as to the nature of the destructive effect of radiation on tissue. The nature of these assumptions will be brought out in what follows. An alternative explanation of the small value of λ_e which does not involve the small-body theory will also be suggested.

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[1] Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1926, c, 390.

II. Primary and Secondary Effects of X-Rays.

The primary effect of x-rays on the light atoms of which living matter chiefly consists is the ejection of a high speed electron from some of them chosen at random in both space and time. Each of these primary electrons ionizes a large number of other atoms by collision before it comes to rest. This secondary ionization appears to be the only effect which we need consider. There is no good reason for supposing that the atom from which a primary electron has been ejected is the seat of any considerable part of the destructive effect; the disintegration of the particular molecule which contains this atom can scarcely be thought of as being more important, in general, than that of any other molecule. There is, on the other hand, plenty of evidence in favor of the view that the destructive effect is associated with the ionization produced by the high speed electron, in comparison with which the ionization by direct absorption of the rays is negligible. In very simple photochemical systems, the reactions produced by x-rays or by α- or β-rays proceed at a rate which is directly proportional to the rate of ionization; it would be rash, of course, to assert that this is true in the case of tissue destruction.

If the x-rays are monochromatic, the primary electrons are all ejected with the same speed and energy; they travel approximately equal distances before stopping, and they knock off about the same number of secondary electrons. The various quantum events or units are, therefore, much alike. Likewise, the events which consist in the production of the various secondary electrons are alike in one respect at least—all of the electrons have the same properties.

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[2] Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1924, xcvi, 207.

Doubtless one or the other of these units corresponds to the hit mentioned above. In what follows, I shall refer to the release of a secondary electron as an *electron-hit*, and to the emission of a primary electron wth its attendant phenomena as a *quantum-hit*. The units of destructive effect dealt with in Crowther's analysis will be called *effective hits*.

III. Some Quantitative Estimates.

Crowther used the K radiation of molybdenum, the α lines of which have a mean frequency of 4.23×10^{18} per second. The primary electron is thus ejected with an amount of energy, $h\nu$, equal to 2.76×10^{-8} ergs. Dividing this by 5.5×10^{-11} ergs, the work required, on the average, to release a secondary electron in air according to Rutherford,[3] we find that each primary electron releases about 500 secondary electrons. This number will be denoted by E_q.

The fourth power law, together with Whiddington's[4] constant for air, shows that the maximum path length of the primary electron in air must be about .2 cm. The value taken directly from one of Sadler's[5] curves is .22 cm. Evidently the law holds nicely even for these very soft rays. In tissue, assumed equivalent to air of unit density, the maximum path, L, is, therefore, about 2.6×10^{-4} cm.

The paths of the primary electrons are, in general, not straight, and, in consequence, the distance in a straight line from the beginning to the end of the path is generally less than L. Consider a plane layer of air, the thickness of which, x, is uniform and somewhat less than L. If a great number of electrons enter this layer through one of its faces, all with the same speed, but in all possible directions, some of them will emerge from the opposite face with some part of their original energy. A fraction, then, of the energy which goes into the layer on one side comes out on the other side. Sadler[5] has shown that the value of this fraction is given by $e^{-\mu x}$ in which μ is the mass-"absorption" coefficient of air for electrons of a particular initial speed. For molybdenum K electrons, he gives 1.18×10^4 as the value of μ. μ does not depend to any great extent on the nature of the absorbing material; we may say with safety that it has the same value for air and for tissue. This is the assumption on which we have already computed L.

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[3] Rutherford, E., Radioactive substances and their radiations, Cambridge, 1913, 159.

[4] Whiddington, R., *Proc. Roy. Soc. London, Series A*, 1911–12, lxxxvi, 360.

[5] Sadler, C. A., *Phil. Mag., Series 6*, 1910, xix, 337.

The exponential law is, of course, not strictly true; it is inconsistent with the existence of a maximum path length. When the absorbing material reaches a thickness such that only those electrons which have travelled very nearly in a straight line can get through, a slight increase in the thickness will stop them all.

The distribution of secondary electrons along the path of the primary electron must now be considered. In any small part of the path, they are distributed very nearly at random—as nearly as the fine structure of matter permits. The space rate of ionization increases, however, as the velocity decreases. At the end of the path, the ionization is probably very intense. Glasson[6] states that over a considerable part of the path, at least, this rate (the number of electrons per cm.) varies inversely as the square of the velocity of the primary electron. This law may, of course, be derived directly from the fourth power law. On this basis, a simple calculation, which need not be given here, shows that by the time the primary electron which we are considering has reached the middle point of its path, it has released 150 secondary electrons, and that, at this point, the mean distance between consecutive electrons is about 7.3×10^{-7} cm. This is enough to show that we are not to think of the secondary ionization as being almost wholly confined to a small region near the end of the path.

Friedrich's *e*-unit of radiation is the amount required to release in 1 cc. of air at N.T.P. 1 electrostatic unit of charge of either sign, or 2.1×10^9 electrons. The mass absorption coefficient of tissue is the same as that of air and it is reasonable to assume, as Crowther does in his earlier paper, that the number of secondary electrons *per quantum* is the same. Since the tissue with which we are dealing is approximately of unit density, an e dose corresponds to the production of 1.63×10^{12} secondary electrons per cc. of tissue. This number will be called E_e.

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[6] Glasson, J. L., *Phil. Mag.*, *Series 6*, 1910, xxii, 647.

IV. The Small-Body Theory.

That λ_e has been found to be very small shows that very few of the hits received by the animal, whether electron-hits or quantum-hits, are effective. From the fact that the atoms from which the high speed electrons are ejected are distributed at random in space, it follows that the probability that a high speed electron will be ejected from within any small portion of the animal is directly proportional to the volume of the portion considered and independent of its position. The same is true of the probability that a secondary electron will be released within the portion considered, provided, of course, that the volume is such that the electron-hits occur independently of one another. Assuming that the destructive effects are confined to some small body within the animal, we may assign volumes to this body such that either of these probabilities will assume any desired value,—in particular the value 5.9×10^{-4} in which case every hit within the small-body will be effective. On the hypothesis that the electron-hit corresponds to the unit of destructive effect, the diameter of the body (assumed approximately spherical) must be about 8.8×10^{-6} cm. Similarly on the quantum-hit hypothesis, the diameter is about 7.0×10^{-5} cm.[7]

Let us now inquire whether this theory is consistent with the postulates on which the statistical treatment of the problem is based. The postulates are: (1) that all effective hits are equally effective, and (2) that λ_e is constant.

Let us consider first the bearing of the electron-hit hypothesis on the small-body theory. L, the path length of the primary electron, is 29.4 times the diameter of the small-body appropriate to the hypothesis that the individual secondary electron corresponds to a unit of destructive effect. Since 500 such electrons are released by the primary electron in traveling a distance equal to L, it is evident that in most cases, in which a high speed electron traverses the small body, it will release more than one secondary electron inside it. Those events, then, which are at random in time, are not the electron-hits at all, but rather showers of electron-hits, and the number of electrons per shower must vary within very wide limits, because the intensities of ionization, at the beginning and the end of the path, respectively, differ so much, and because the length of the path through the body varies from zero to the length of the diameter,—even more if the path is not straight. The average number of electrons per shower is, of course, very great in the case of primary electrons which enter from outside and come to rest inside the body; and correspondingly small for those which are ejected from within the body. For primary electrons which pass through the body, the average number of secondaries per shower is about 11, since the mean length of a great number of straight paths through a sphere, chosen at random, is equal to two-thirds of the diameter. Electron-hits are not then at random in time—not even approximately so—and postulate 2 is not fulfilled.

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[7] As the result of an error in calculation, which Dr. Crowther discovered after publication, the diameters assigned to the body in his paper differ somewhat from those given above.

It is apparent then that we must abandon either the electron-hit idea or the small-body theory.

We have now to deal with the quantum-hit hypothesis in its relation to the small-body theory. Let us assume for the sake of the argument that the distribution of the destructive effect along the path of the primary electron is the same as that of the secondary ionization—which would be true if we were dealing with a simple photochemical system. It is evident that some of the primary electrons, ejected from atoms inside the small-body, must escape from the body with a considerable part of their initial energies. Likewise, other high speed electrons, ejected from matter outside the small-body, will enter it before coming to rest. In these cases, the effectiveness of the hit will be less than in the cases in which the whole path lies inside the body. Hits of this kind will be referred to in what follows as "partial" hits.

The relative number of partial hits cannot be so small as to be negligible. On the quantum-hit hypothesis, the diameter of the small-body is 7.0×10^{-5} cm., whereas L is 2.6×10^{-4} cm., *i.e.* 3.7 times the diameter. In order to make a very rough estimate of the relative importance of the partial hits, we set x equal to 3.5×10^{-5} cm., the radius of the sphere, in the expression $e^{-\mu x}$ discussed in Section III, and we find that, of the energy associated with high speed electrons ejected from points midway between the faces of a layer of tissue of thickness equal to the diameter of the small-body, 66 per cent escapes from the layer. If we say that 66 per cent of the electrons escape, we shall make an underestimate, for each of the escaping electrons has lost a part of its initial energy. If we say that 66 per cent of the high speed electrons, ejected from the center of the small-body, escape, we shall underestimate the number still further, because the radius of the sphere is much smaller than the mean of the distances between a point in the middle of the plane layer and the points where the electrons escape from the surfaces of the layer.

Of the high speed electrons ejected from the center of the small-body, then, at least 66 per cent escape; of those ejected from points near the surface of the body, at least 50 per cent escape. Let us say that at least 50 per cent of all high speed electrons released within the small-body escape from it. Now for every one which escapes, another enters from outside. The whole number of hits, both total and partial, *i.e.* the whole number of those events which occur at random in time, is then increased by 50 per cent and two-thirds of them are partial hits. To keep the whole number down to 49, the body must be made smaller, and this will make the relative number of partial hits still greater. From what has been said in Section III about the distribution of ionization along the path it appears that we must give up either the small-body theory or the idea *that the distribution of the destructive effect along the path of the high speed electron is similar to that of the ionization.*

It is conceivable that the destructive effect, though brought about by ionization, is not measured by it; that it is conditioned in some way by the density of ionization or otherwise. It might be supposed, for example, that at the end of a path a small portion of tissue is injured so seriously that repairs are impossible; that at other points along the path the injury, being diffuse, is rapidly made good. If this were true and if the permanent injury which corresponds to an effective hit were confined to a very small region—to 1 per cent, let us say, of the path length, no objection could be raised against the small-body theory.

This idea, that the hit is localized in some very small part of the path, is the assumption referred to in the introduction. It should be noted that it amounts to something more than the assumption that some single molecule, peculiarly essential to the organism, happens to lie in the path of the primary electron and to be destroyed by it; if this were the case, the introduction of the small-body theory would no longer explain why all quantum-hits are effective and equally effective. Whether or not the difficulty of reconciling this assumption with the known facts of photochemistry is more than sufficient to compensate for the usefulness of the small-body theory is a matter of personal judgement.

V. An Alternative Hypothesis.

If the small-body were subdivided into a great number of much smaller bodies, and if these smaller bodies were placed as far apart as possible, the probability that a quantum would make two or more effective electron-hits would be made smaller. To make it negligible, however, the bodies would have to be very small in comparison with the mean distance between consecutive electrons in a shower. This suggests that an effective hit may correspond in some way with the destruction of molecules of a certain kind or kinds distributed throughout some considerable part of the tissue. The number, N, of such molecules present in the animal at the beginning of an exposure would have to be very great, of course, in comparison with n, which is 49, otherwise λ_e would become appreciably smaller as more and more effective hits were made. That the loss of so small a fraction of these molecules should have so profound an effect suggests either that they are essential parts of some structure or that the destruction of the molecule is followed by a recombination of the component atoms to form a molecule of a new substance which is highly toxic. The postulate that all effective hits must be equally effective seems to favor this latter idea, and to require that we restrict ourselves to one kind of molecule. We shall consider, then, that the making of a molecule of the toxic substance Y constitutes an effective hit. It appears highly improbable that the destruction of a molecule of substance X would always result in the production of a molecule of Y; it would certainly be more reasonable to suppose that Y is formed only when X loses certain particular electrons. The problem cannot be analyzed, of course; too little is known about the fine structure of matter. In what follows, an attempt will be made to estimate the various quantities involved in the relatively simple case where Y is formed when X, represented by an idealized molecule, loses one particular electron. It will be assumed that in a microscopic sense the molecules of X are at all times distributed at random in space insofar as the finite size of the molecule permits,—the arrangement to be expected in a solution. It will appear further on that the molecule would have to be extremely large to have an appreciable effect on this distribution; it will be assumed tentatively that it has none.

Multiple Effective Hits Made by One Quantum.

Let us assume for the moment that, in the ordinary sense, X is distributed uniformly throughout the whole volume of the animal. If the probability that a quantum, falling entirely inside the animal, will make an effective hit be represented by p; and if V be the volume of the animal, then

$$p = \frac{\lambda_e E_q}{E_e V}. \tag{1}$$

V may be taken as 10^{-7} cc. Using the value of λ_e given by Crowther, and the values of E_q and E_e found in Section III, we find that $p = 1.8 \times 10^{-6}$. Now it is not the quantum as a whole, but rather the individual secondary electrons which correspond to the hits. In the language of probability we may, therefore, speak of the number of "trials" per quantum. If the molecule of X were so very small that it would never lose two or more electrons, the number of trials would be equal to E_q. If the molecule were larger, the number of trials would be less than E_q. When p is less than 1, a decrease in the number of trials, corresponds to a decrease in the ratio of p_r to p_1; p_r being the probability that the quantum will make exactly r effective hits and p_1 the probability of exactly one such hit. For example, if the number of trials were 1, the probability of a multiple hit would be absolutely zero. To find the maximum value of this ratio, which we may call R_r, we take the number of trials as infinite in which case R_r is $p^{r-1}/r!$ In particular, R_2 is $p/2$ or 9.0×10^{-7}.

R_r is so small that we may now reconsider the assumption that X is distributed uniformly throughout the whole volume of the animal. Other things being the same, p is proportional to the number of molecules of X per unit volume in the region where the quantum falls. If in some part of the animal the concentration of X were 1000 times as great as the mean concentration, then in this part p would be 1.8×10^{-8}; only about one effective hit in a million would be a "double," and one in less than 10^{12} a "triple" hit. p, for a particular quantum, cannot, of course, be greater than the value corresponding to the maximum concentration which the primary electron encounters; the concentration may change from point to point, therefore, as abruptly as desired.

It is evident, then, that effective hits are at random in time and that the molecules of X to be hit effectively are chosen at random, even though no unreasonable restrictions are placed on the way in which X is distributed.

The Size of the Molecule.

We have now to deal with the slow change in λ_e which takes place in consequence of the fact that N is finite. Let P_1 be the probability that a destroyed molecule of X, chosen at random from among the whole number of those that have been hit, will have lost exactly one electron; and let P' be the probability that a molecule, chosen at random from among all those which have lost exactly one electron, will have lost the particular electron required. In the normal case, the making of n effective hits corresponds to the destruction of n/P_1P' molecules of X, and, therefore, n/NP_1P' represents the relative change in λ_e. This latter quantity must then be small; just how small is a matter of judgment. It ought certainly to be smaller than the errors in experiment, and the results of Crowther's experiment fit the theoretical curve very nicely. The values of P_1 and P' depend on the properties of the molecule of X. As the volume, v, and the complexity of the molecule increase, both P_1 and P' diminish; furthermore, since Nv may not be greater than the whole volume of the animal, the maximum value which we may assign to N diminishes. The hypothesis is, therefore, consistent for a given value of v provided the corresponding value of NP_1P' is sufficiently large in comparison with and provided the value of P_1P' appropriate to a molecule of volume v is not so small that N has to be greater than V_-/v. It is obvious that these conditions are more easily fulfilled the smaller and simpler the molecule. We have to find out, if possible, whether or not they are fulfilled when the molecule is fairly large.

In order to estimate P_1 and P', it is necessary to make certain idealizing assumptions as to the nature of the molecule and to assign a definite size to it. To make it possible to treat P_1 statistically, it is assumed that the molecule will behave as though its electrons were distributed at random inside a sphere, the volume of which is the same as that of the molecule; the probability of releasing an electron being the same for all of them. For convenience, the diameter of the sphere is set equal to 10^{-7} cm. The volume is then equal to that of the molecule of oleic acid according to Langmuir.[8]

In the case of a complex organic molecule the electrons must be fairly evenly distributed throughout what we call its volume, *i.e.* the room which it occupies when stacked with other molecules to constitute matter in the solid state. Such a distribution, together with the movements of the electrons, and the random orientation of the molecule with respect to the path of the high speed electron may reasonably be thought of as equivalent to a random distribution. The probability that an electron will be released from the molecule is then directly proportional to the path length through the molecule. The constant of proportionality will be nearly enough equal to that for tissue in general, if we assign to the molecule the same number of electrons as that in the molecule of oleic acid, *i.e.* 158. P' will then be .0063. It will appear presently that, for a molecule of this size, the conditions imposed by the size of the animal and the desired constancy of λ_e are fulfilled with a margin of safety which is so great that the errors involved in idealizing the molecule need not be small.

We must now try to estimate P_1. Since the particular electron to be removed may be anywhere, we must suppose that it is in the worst place, *i.e.* at the center of the sphere. It will have the same chance of being hit wherever it is, but if it is at the center, the primary electron must traverse the longest path through the sphere to reach it, and the probability of removing two or more electrons from the same molecule increases with the path length. It has been shown in Section III that, on the average, 30 per cent of the ionization, *i.e.* 150 secondary electrons, lie in the first half of the path of the high speed electron, and that even at the midpoint of the path, consecutive secondary electrons are no closer together on the average than 7.3×10^{-7} cm., which is over seven times the maximum path length through our molecule. Let us confine our attention to the first half of the path for the moment. If the high speed electron were shot into a solid mass of X, the molecules being lined up in such a way that it would traverse a diameter of each, only about one molecule in seven at the midpoint of the path would lose an electron. When we remember that electrons are released farther and farther apart as we go from the midpoint toward the beginning of the path, we see that the number of cases in which a molecule loses two or more electrons must be very small in comparison with the number of those in which it loses only one. We shall make no great error if we assume that all hits in the first half of the path are "single hits." There are, undoubtedly, many single hits in the last half of the path, where the ionization is more intense, but we shall ignore them in order to make sure that we are not over-estimating P_1. The total number of single hits is then equal to 150, the number of electrons in the first half. It should be remembered that we assumed that the path follows the diameter of the molecule for the purpose of estimating the relative number of double hits. The number of single hits just found, 150, has nothing to do with the exact location of the path.

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[8] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

Now P_1 is, in the long run, the ratio of the number of molecules which have lost one electron to the total number destroyed by the loss of any number of electrons. For the average quantum, falling in a mass of X in the pure state, the whole number of molecules destroyed, which we will denote by M, must be less than 500, for some of the molecules lose two or more electrons. If all of the electrons in the last half of the path were lost by the same molecule, an absurd assumption, M would be 151, and P_1 would be 1. If all of the hits in the second half of the path were doubles, M would be 325 and P_1 would be .46. This is the minimum value of P_1. To sum up—P_1 lies somewhere between .46 and 1, and M lies between 150 and 500. Even though the value of P_1 depends in part on M, we must consider the limiting values separately.

λ_e, the probability per e-unit of making an effective hit, is given by

$$\lambda_e = \frac{E_e}{E_q}\cdot N v M P_1 P'. \tag{2}$$

Substituting the limiting values of M, found above, we see that NP_1P' must lie between 6.9×10^5 and 2.3×10^6. To be on the safe side, we use the smaller of these numbers to test the constancy of λ_e. $n \div NP_1P' = 7.1 \times 10^{-5}$. In the normal case, λ_e may change, then, by as much as .007 of 1 per cent. Such a change is too small to consider.

Now we consider the maximum value of N. To make N as large as possible, we divide the greater value of NP_1P', which is 2.3×10^6, by the minimum value of P_1P', which is $.46 \times 6.3 \times 10^{-3}$, and N comes out to be 8.0×10^8. The total volume of X in the animal is then 4.2×10^{-13} cc., which is only 4.2×10^{-6} times the volume of the animal. The "volume" concentration of X is then only .0004 of 1 per cent; it is, of course, so small that cases will be very rare in which the finite size of the molecule interferes with the assumed random distribution.

The margins of safety in the variation of λ_e and in the total volume of substance X are obviously so great that the error involved in assuming that the real molecule behaves like the ideal one may also be very great without rendering the general hypothesis untenable. There can be little doubt that, if an effective hit corresponds to the removal of a particular electron from a molecule of X, the molecule of X may be fairly large and complex.

VI. CONCLUSION.

If we accept the idea that the reactions of living matter to x-rays are the result of ionization, we find that Crowther's small-body theory serves to explain the small value of λ_e only provided it be assumed further that the unit of destructive effect which corresponds to an effective hit is associated with the quantum and that it is localized in a region the dimensions of which are very small in comparison with the path length of the high speed electron.

It is suggested that there exists in the animal a substance, X, distributed throughout a considerable part of the tissue, a molecule of which turns into a molecule of a substance Y when it loses a particular electron, and that the formation of a molecule of substance Y constitutes an effective hit. This hypothesis seems to be consistent if the molecule of X is not too large. There is, of course, no good reason for supposing that it is the true explanation of the phenomenon. It is put forward merely to show that we may accept the theory that the variations in reaction are inherent in the x-ray itself without accepting the small-body theory.

VII. SUMMARY.

1. The theory which Crowther has advanced to account for the variation of the lethal dose of roentgen rays among the individuals of a group of *Colpidium colpoda* is reviewed.
2. It is shown that the use of his small-body theory to explain the small value of λ_e leads to certain further assumptions about the nature of the destructive effect.
3. An alternative hypothesis is discussed.

TIME RELATIONS OF GROWTH.

III. GROWTH CONSTANTS DURING THE SELF-ACCELERATING PHASE OF GROWTH.*

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(Accepted for publication, January 31, 1927.)

I.

INTRODUCTION.

The period of growth of multicellular organisms, and of populations of organisms, may be divided into two fairly distinct phases: (1) a *self-accelerating phase* during which the time rate of growth increases with the increase in size of the organism or the population; and (2) a *self-inhibiting phase* during which the time rate of growth decreases with the increase in size of the organism, or population. The question of mechanisms bringing about the general similarity in the course of growth of animals, plants, and populations, need not be gone into in this place except to note that the course of growth is in all these cases governed, directly or indirectly, by the same two primal forces: (1) the force inherent in all organisms to reproduce at a constant percentage rate; and (2) the growth-inhibiting forces resulting from the finite nature of the universe in which the organisms find themselves.

The purpose of this series of papers is merely to present quantitative analyses of growth curves, with special reference to developing methods for computing *rational* growth constants. The first two papers[1] were concerned with the self-inhibiting phase of growth, that is the phase of growth following the major inflection in the time curve of growth. This paper is concerned with the *self-accelerating phase* of growth.

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* The principal portions of this paper have been presented before the Physiological Section of the Botanical Society of America, in Philadelphia, December 30, 1926.

[1] Brody, S., *J. Gen. Physiol.*, 1925-27, viii, 233. Brody, S., Sparrow, C. D., and Kibler, H. H., *J. Gen. Physiol.*, 1925-26, ix, 285.

II.

Proposed Methods for Computing Growth Rates.

Four methods have been proposed for computing growth rates.

1. The Method of Minot.—Minot computed the rate of growth by dividing the gain in weight during a finite unit of time by the weight at the beginning of the unit of time, as represented by the equation

$$R = \frac{W_2 - W_1}{W_1} \tag{1}$$

or

$$W_2 - W_1 = R\,W_1 \tag{1a}$$

in which W_1 and W_2 are, respectively, the weights at the beginning and the end of the unit of time, R is the relative (or when multiplied by 100, the percentage) rate of growth.

Employing this method he was led to the conclusion that, in warm blooded animals, the percentage rate of growth declines from 1000 per cent per day shortly after fertilization, to 3 to 7 per cent per day at the time of birth or hatching.

There is this objection against the use of equation (1), for the self-accelerating phase of growth: It is based on the assumption that growth is a discontinuous process; *i.e.*, that the increments are added at arbitrary time intervals, t_1, t_2, t_3. . . As a matter of fact, statistically considered, growth is a continuous process, and the relative rate of growth, must, therefore, be represented by the equation

$$k = \frac{dW/dt}{W} \tag{2}$$

or

$$\frac{dW}{dt} = kW \tag{2a}$$

in which k is the instantaneous relative rate (or when multiplied by 100, percentage rate) of growth, corresponding to R in equation (1).

The error introduced by the use of equation (1) is very considerable, as may be seen from the following considerations.

From equation (2), at time t_1, the weight, W_1, of the organism is represented by the equation

$$W_1 = Ae^{kt_1} \qquad (3)$$

At time t_2, it is represented by

$$W_2 = Ae^{kt_2} \qquad (3a)$$

Subtracting the former from the latter we obtain,

$$W_2 - W_1 = Ae^{k(t_2 - t_1)}$$

Dividing by (3),

$$\frac{W_2 - W_1}{W_1} = e^{k(t_2 - t_1)} - 1$$

Transposing and taking logarithms,

$$ln\left(\frac{W_2 - W_1}{W_1} + 1\right) = k\ (t_2 - t_1)$$

For 1 unit of time,

$$k = ln\left(\frac{W_2 - W_1}{W_1} + 1\right) = ln\ (R + 1) \qquad (4)$$

Numerical relations between k, the instantaneous rate of growth, and R, the rate of growth as determined by Minot, may be computed by substituting the values of R in equation (4). The results for a series of substitutions are presented graphically in Fig. 1. Fig. 1 makes it clear that Minot's method (equation (1)) cannot be used for computing the relative rate of growth when the rate exceeds 10 per cent for the unit of time under consideration.

2. The Method of Pearl.—In 1907

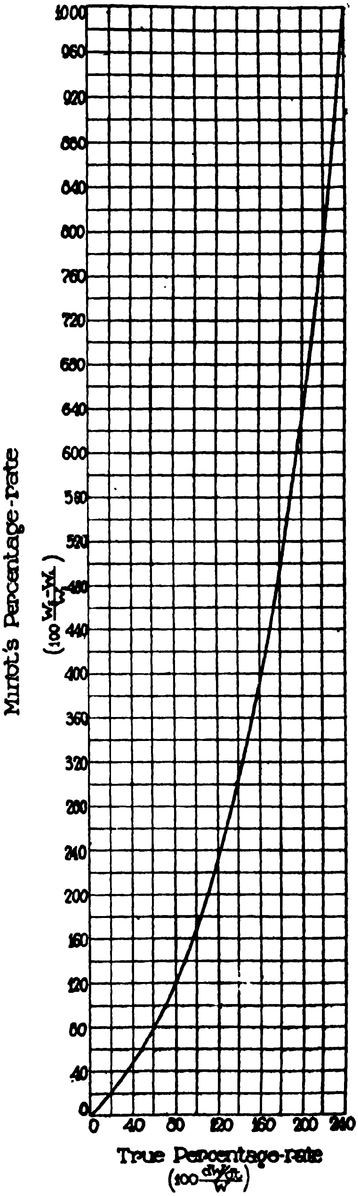

FIG. 1. The relation between the increase in percentage rate as computed by the logarithmic method and by the arithmetical method of Minot.

Pearl proposed the method for computing growth rates represented by the equation

$$\frac{dW}{dt} = \frac{k}{t-a} \tag{5}$$

which in the integrated form is,

$$W = A + k\,ln\,(t-a) \tag{5a}$$

Equation (5) cannot, evidently, be used to represent growth during the self-accelerating phase of growth, inasmuch as the time rate of growth is represented by (5) to decline with time, while during the self-accelerating phase, growth increases with time.

3. *The Method of Robertson.*—In 1908 Robertson suggested that the equation

$$\frac{dW}{dt} = k\,W\,(A-W) \tag{6}$$

or

$$\frac{dW/dt}{W\,(A-W)} = k \tag{6a}$$

may be used to represent certain phases of growth termed by him *growth cycles.* Equation (6) indicates that the velocity of growth is a function not only of the size, W, of the organism, but also of growth yet to be made, $(A\text{-}W)$. The objections against this equation are indicated in the following sections.

4. *The Method of Pearl and Reed.*—Pearl and Reed introduced the following modification in the autocatalytic equation (6), employed by Robertson. They replaced k, by "some as yet undefined function of time," $F\,(t)$, "since the rate of growth of W is dependent upon factors that vary with time." They then assumed that $F\,(t)$ may be replaced by the series

$$k_1t + k_2t^2 + \ldots + k_nt^n$$

thus changing equation (6) into

$$\frac{dW/dt}{W(A-W)} = F\,(t) = k_1t + k_2t^2 + k_3t^3 + \ldots + k_nt^n \tag{7}$$

They found the integrated form of equation (7) to be elastic enough to fit the growth curve of the rat beginning with 10 days after birth. Since, however, the period preceding 10 days after birth is an exceedingly important one, and since the constants in equation (7) do not have definite physical meaning (thus, when $(A\text{-}W) = 1$, $\frac{dW/dt}{W} = k_1t + k_2t^2 + \ldots + k_nt^n$; what is the physical meaning of $k_1t, k_2t^2 \ldots$?) therefore the method of Pearl and Reed is not suitable for the purpose under consideration (which is to evaluate *rational* growth constants; *i.e.*, constants having well defined physical meaning).

III.

The Method Employed in This Paper.

During the self-accelerating phase of growth, when the time rate of growth increases with the increase in the size of the organism, it is reasonable to attempt to relate the time rate of growth, $\frac{dW}{dt}$, to the size, W, of the organism, by the function

$$\frac{dW/dt}{W} = k \tag{2}$$

or

$$\frac{dW}{dt} = k\,W \tag{2a}$$

The first thought is that the constancy of k may be tested by integrating (2) and solving for k

$$k = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

As a matter of fact this is an impractical procedure for two reasons. First, it is not known for how long a period equation (2) represents the data; *i.e.*, $t_2 - t_1$ may represent more than one stage or cycle of growth; second, ratios are very sensitive to slight changes in one or both of the variables. This fact taken with the large experimental errors involved in this work, makes the results apparently erratic.

A better method is to plot the logarithms of the size, or weight, of

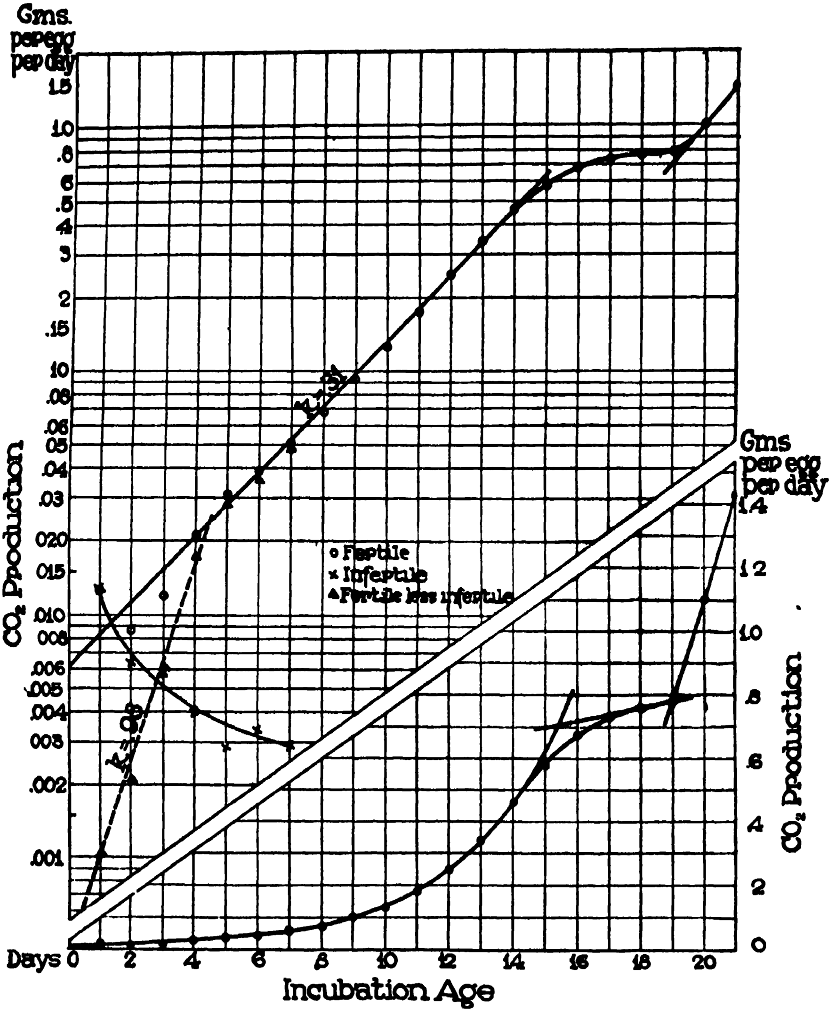

FIG. 2. The course of carbon dioxide excretion in the chick embryo with advancing age plotted from data by Atwood and Weakley. From 0 to 4 days, the instantaneous percentage rate of growth appears to be 98 per cent per day (the amount of carbon dioxide excretion is doubled once in .7 day, or once in 17 hours); between 4 and 14 days, the rate of increase in carbon dioxide excretion is 31 per cent per day (it is doubled once in 2.2 days). The pause in the curve coincides with the maximum in the mortality curve (of Fig. 3), and with the change in the mode of respiration (see text).

the organism, against age, since the integrated form of (2) may be written

$$W = Ae^{kt}$$

Therefore

$$\ln W = \ln A + kt$$

If the data points of the logarithms of weights plotted against age are distributed about a straight line, then the percentage rate of

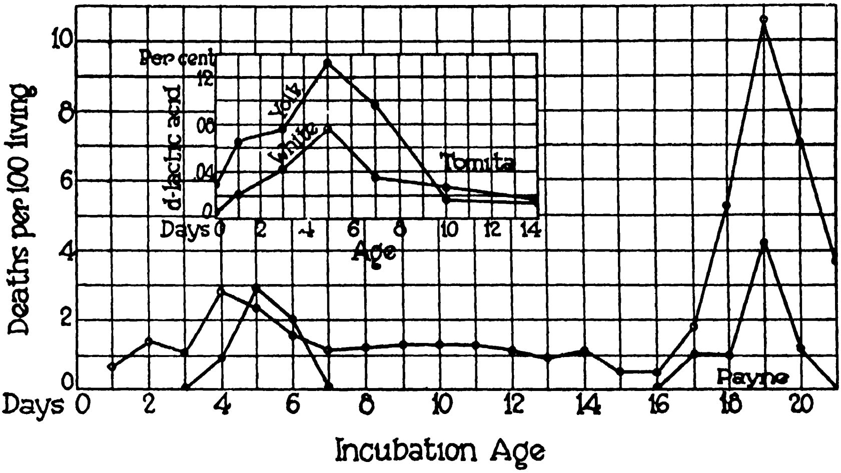

Fig 3 Age changes in the percentage mortality with increasing age in the chick embryo Open circles represent mortality data of embryos incubated in an incubator, solid circles represent incubation under hens The first peak in the mortality curve corresponds with the peak in the concentration of lactic acid as found by Tomita The second peak in the mortality curve coincides approximately with the pause in the growth curves (Figs. 2 and 5)

growth, represented by 100 k, is constant, and k is the growth constant we are seeking. Instead of plotting logarithms of weights, we may plot the data on paper on which the axis of ordinates is divided logarithmically (*i.e.* on arithlog paper).

IV.

The Results of Plotting Growth Data on Arithlog Paper.

Fig. 2 represents the course of increase in carbon dioxide excretion with age in the chick embryo, as plotted on arithlog paper. The rate

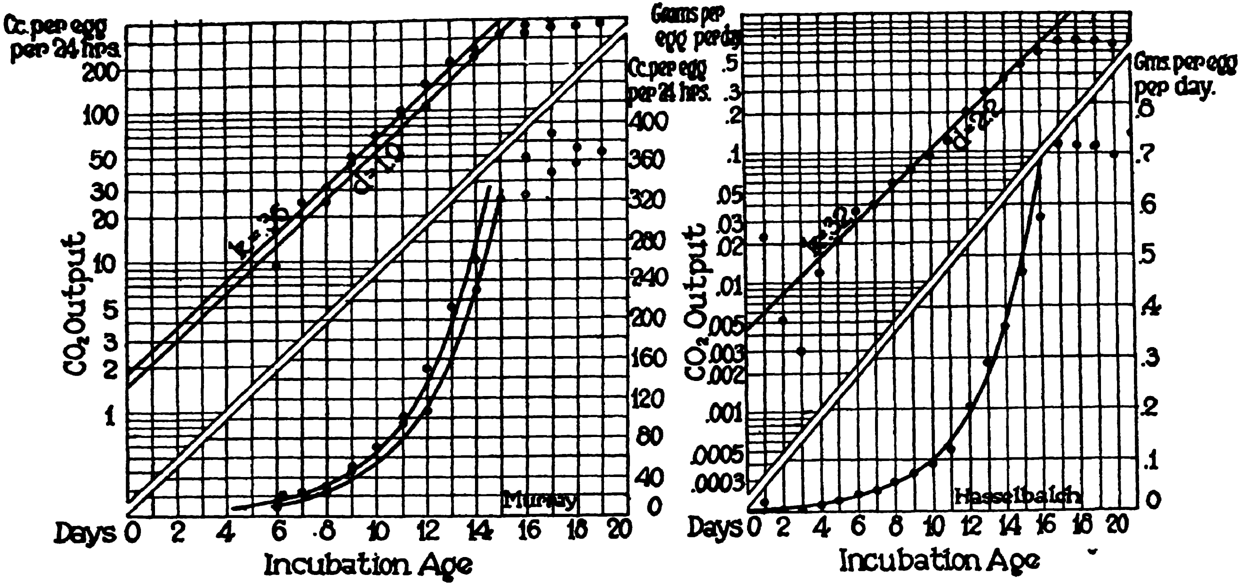

FIG. 4. The course of carbon dioxide excretion in the chick embryo, plotted from data by Hasselbalch and Murray. *d* refers to the time in days in which the magnitude of the carbon dioxide excretion is doubled.

of growth, as measured by the increase in carbon dioxide production, is constant between the 1st and 4th day of incubation. The increase is of the order of 100 per cent per day, and not 1000 per cent as postulated by Minot. From the 4th to the 15th day, the increase is likewise constant; it is 31 per cent per day.

The pause in the curve between 17 and 19 days, is, no doubt, associated with the change in the mode of respiration (from the aquatic to the terrestrial mode) which takes place at this time. Fig. 3, representing the course of mortality, likewise presents a disturbance at this time. The peak of mortality at 5 days also represents a critical period as indicated by the presence of a peak in the lactic acid curve.

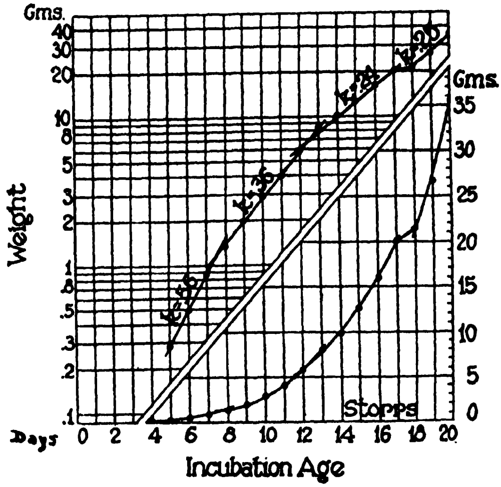

FIG. 5. Growth in wet weight of the chick embryos plotted from data by Lamson and Edmond (Storrs Agricultural Experiment Station). The curve appears to consist of four segments each of which represents growth at a constant percentage rate. The pause in the curve between 17 and 18 days coincides approximately with the second peak in the mortality curve (Fig. 3). Growth at an instantaneous rate of 56 per cent per day indicates that the body weight is doubled once in 12 days; at 36 per cent per day, the body weight is doubled once in 1.9 days; at 24 per cent per day, the body weight is doubled once in 29 days.

These results are quite unexpected and no doubt, new. Students of animal growth have accepted the notion of Minot that the percentage rate of growth declines in a continuous manner with age. The breaks in the curves substantiate, in a way, the "human metamorphosis" conception recently advanced by Davenport.

Fig. 4 represents the course of carbon dioxide production plotted from data by other investigators. The differences between the curves in Figs. 2 and 4 are due to differences in experimental procedure.

Figs. 5, 6, and 7 represent the curves of growth in weight of the chick embryo. There are differences between the weight and the carbon dioxide curves, which leave room for discussion. There are also differences between the weight curves as plotted from data by different investigators. These differences are probably due to differences in the experimental technique employed, especially differences in incubation temperatures. That differences in temperature bring about changes in the growth rates, especially in the earlier stages of incubation, is illustrated by Fig. 8.

During postnatal life, the fowl grows at 5 per cent per day up to 3 weeks, and at 3 per cent from 3 to 12 weeks. The major inflection in the curve takes place at the age of about 12 weeks.

It may be noted in this connection that the rat, guinea pig, cow, sheep, and probably other domestic animals, grow at approximately the same percentage rate during the juvenile period (the stage preceding the major inflection); namely, 2 to 3 per cent per day. Man, however, grows during this period at quite a different (much lower) percentage rate.

The results obtained with the domestic fowl were practically duplicated, as far as the available data permitted, with the rat, guinea pig, cow, sheep, and domestic pig. The data of the rat are of special interest on account of the break in the curve at birth as shown in Fig. 9. It is probable that there is a break in the curve at birth in all classes of animals.

The curve of man differs in several important respects from the curve of animals. The curve of man requires a more extensive discussion than can be given at this time. For this reason a separate paper will be devoted to the growth curve of man.

The curve of plants is similar to that of animals. However, the inadequacy of the data and the relatively large experimental errors

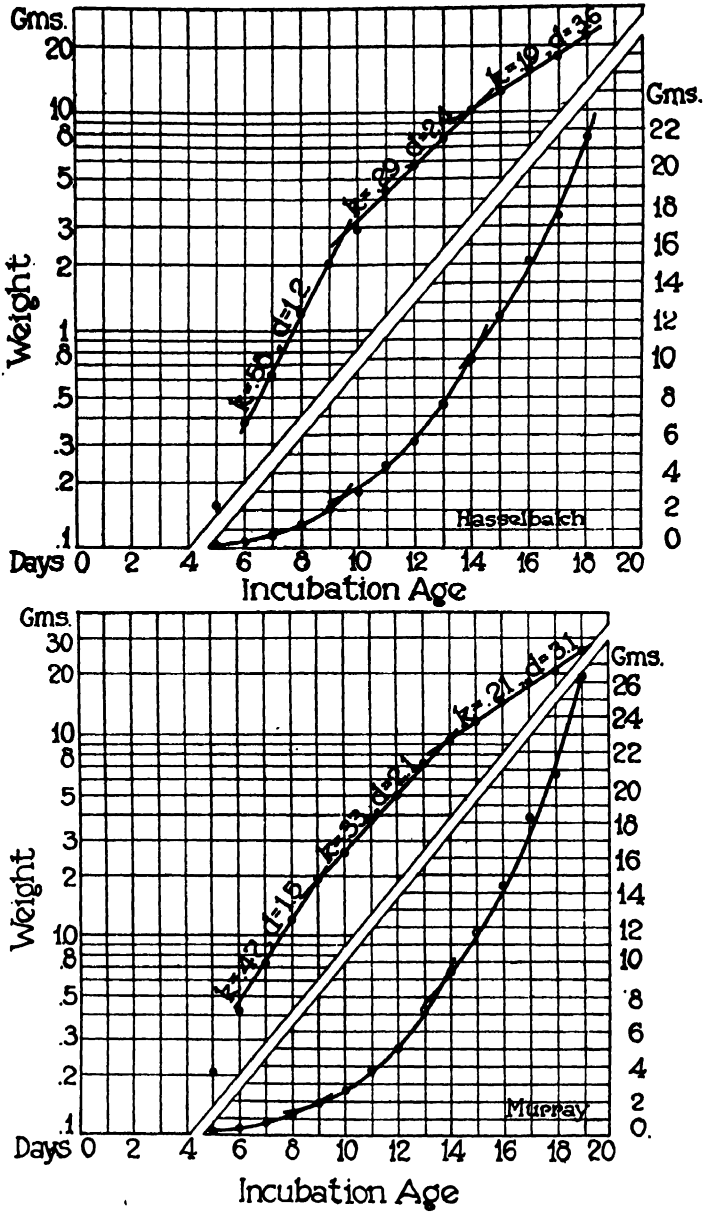

FIG. 6. Growth in wet weight of the chick embryo, plotted from data by Hasselbalch and by Murray. The value of 100 *k* represents the instantaneous percentage rate of growth per day. The values of *d* represent the time in days required for the body to double its weight.

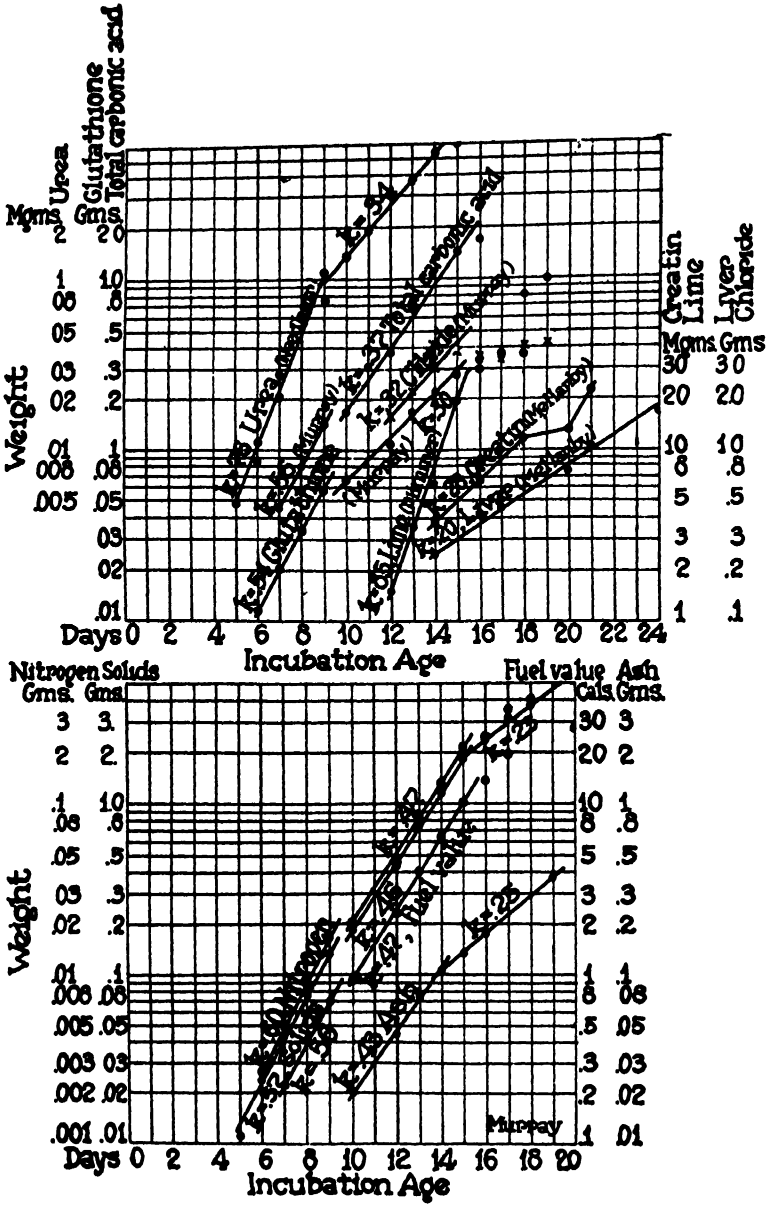

FIG. 7. The course of increase in chemical constituents in the chick embryo with advancing age. The sources of data are indicated on the chart (compare with Figs. 5 and 6).

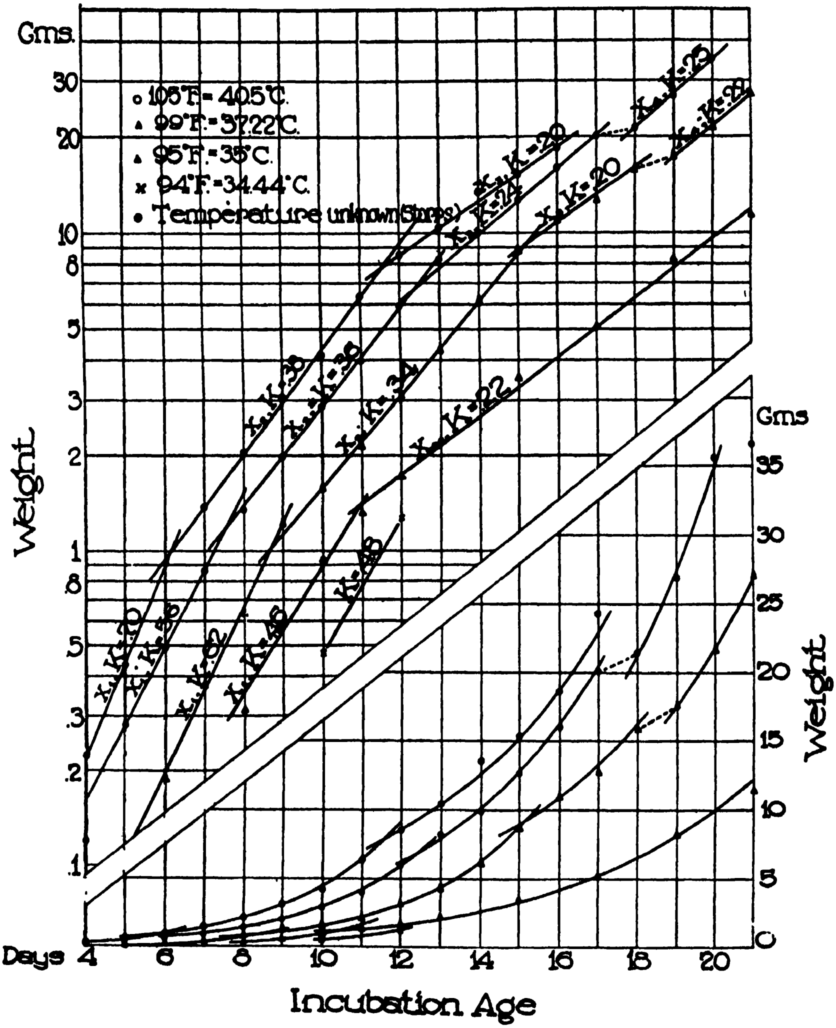

FIG. 8. The effect of temperature on the course of growth of the chick embryo (E. W. Henderson and S. Brody).

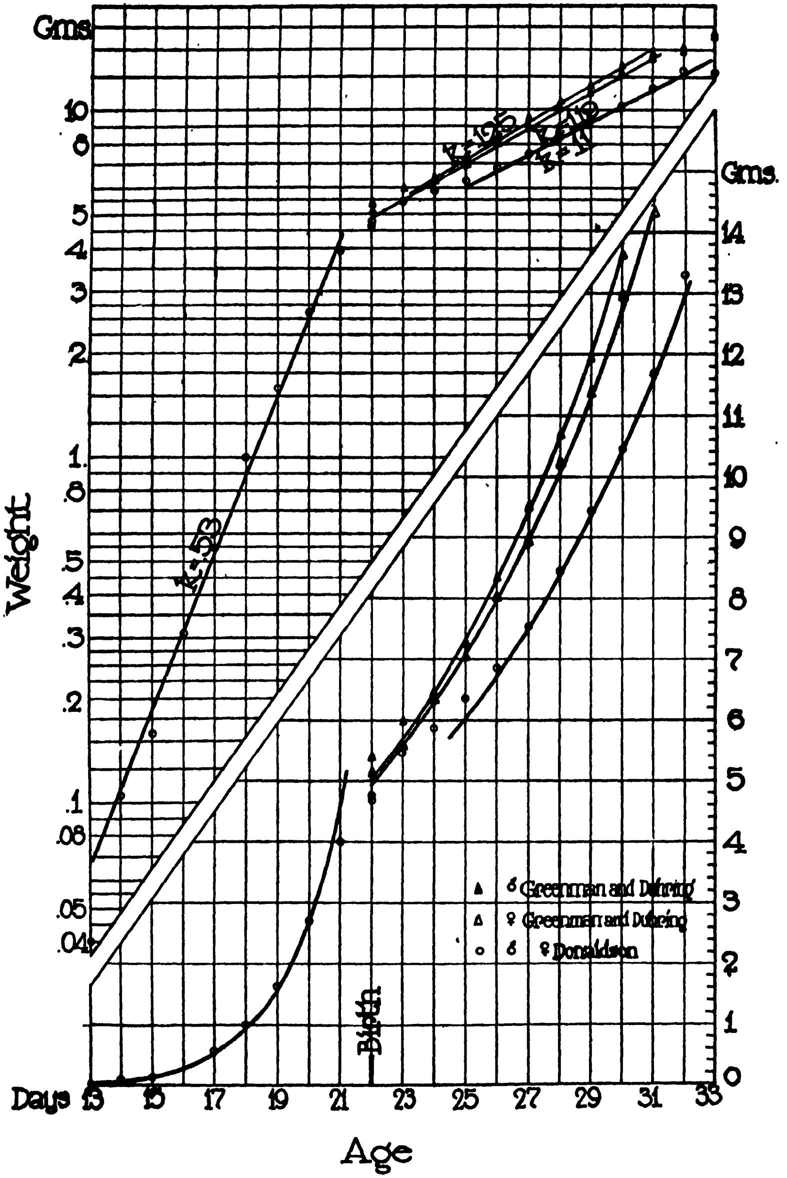

FIG. 9. The course of growth of the white rat plotted on arithlog and on coordinate paper. On the arithlog paper the data points are distributed around a straight line indicating that the percentage rate of growth is constant. There is an abrupt break in the curve at the time of birth and the percentage rate is seen to drop from 53 to about 12 per cent. Data preceding birth by Stotsenburg.

involved in the investigation of plant materials do not permit formulating conclusions as definite as with animals.

Fig. 10 shows the course of growth of the wheat kernel. The development of the seed corresponds, in time, to the prenatal growth in animals. However, it so happens that in the wheat kernel the embryo is a small fraction of the whole seed (about one-thirteenth), and so the data represent more than embryonic growth.

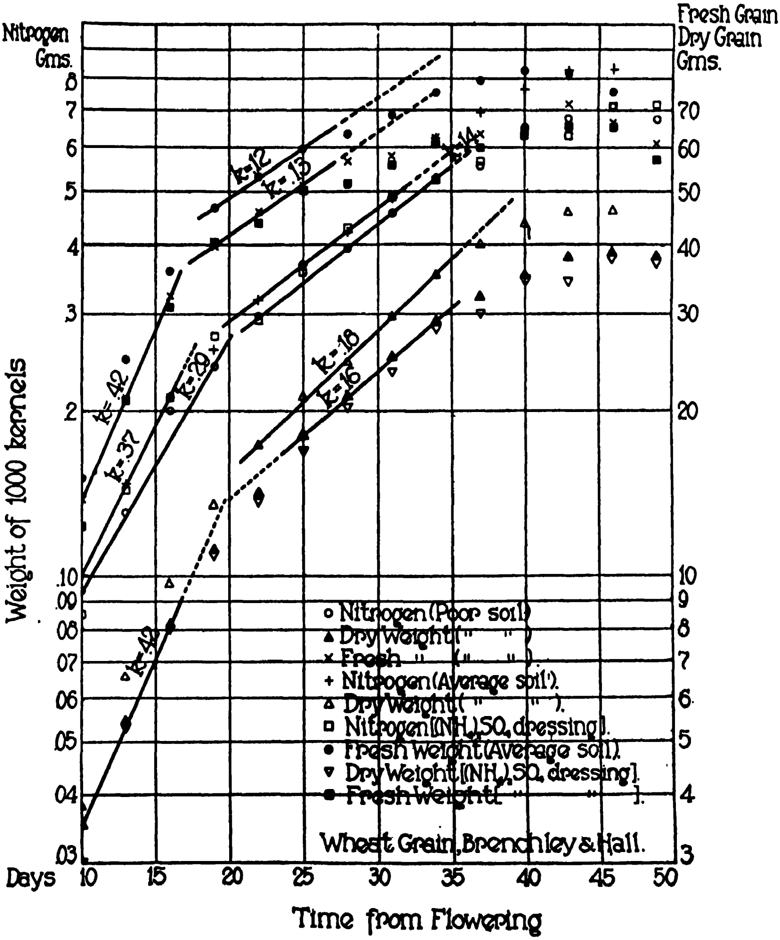

FIG. 10. Growth of the wheat grain. *k* and *d* have the usual meaning.

Fig. 11 represents the period of independent growth of the maize plant. The segment preceding flowering corresponds to the juvenile period in animals, and as in animals, the percentage rate of growth is constant. The inflection occurs at the time of flowering, which corresponds to puberty in animals The major inflection in the curve invariably occurs at the time of flowering in higher plants, and at puberty in higher animals.

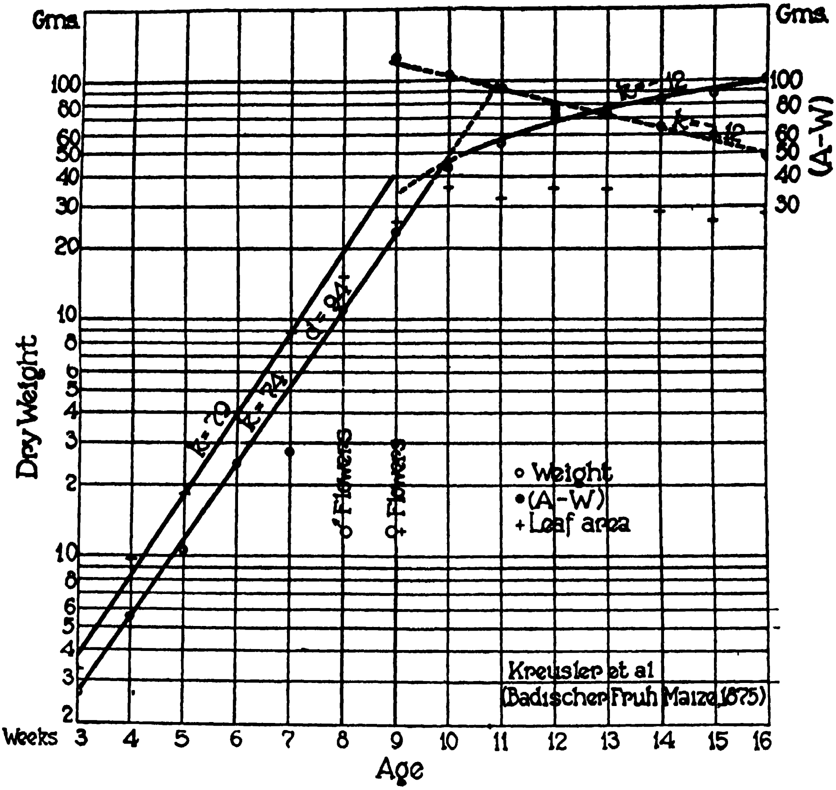

Fig. 11. The course of growth of the maize plant (note that the week is the unit of time in this case).

Figs. 12 and 13 represent, respectively, the growth of bacterial and human populations. The rate of growth is constant during the period preceding the major inflection.

As to the bearing of this work on the problem of *growth cycles*, the situation, as it appears to the writer, is as follows: All curves pass through an inflection which joins the strictly self-accelerating phase with the strictly self-inhibiting phase of growth. For this period,

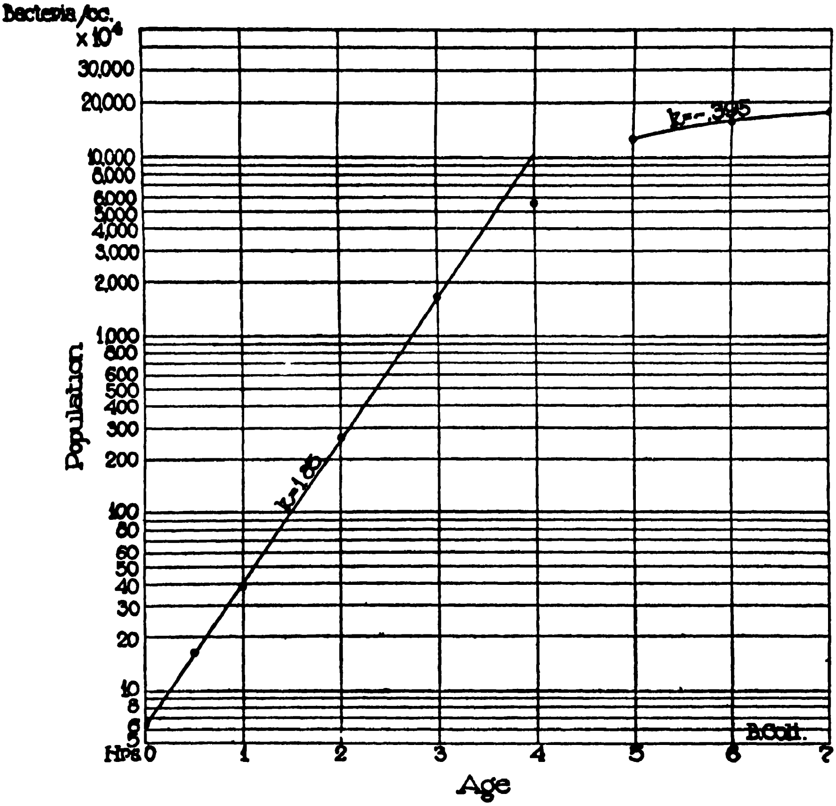

FIG. 12. The course of growth of *B. coli* at 37°C. in a given volume of broth (data from Experiment 7 of McKendrick and Pai). The value of k, 1.85, indicates the population of bacteria increases at 1.85 per cent per hour. That is, the population doubles itself every .69/1.85 = .37 hours, or 22.2 minutes. Following the 5th hour, the percentage rate of growth is constant with respect to the growth yet to be made.

which is relatively short, equation (6), the "autocatalytic" equation of Robertson, or the "logistic" equation of Pearl, can be fitted satisfactorily, especially, if a constant, or constants, is employed to compensate for the asymmetric nature of the curve. This equation cannot, however, be satisfactorily fitted to the infantile (except in man), or to the juvenile cycle.

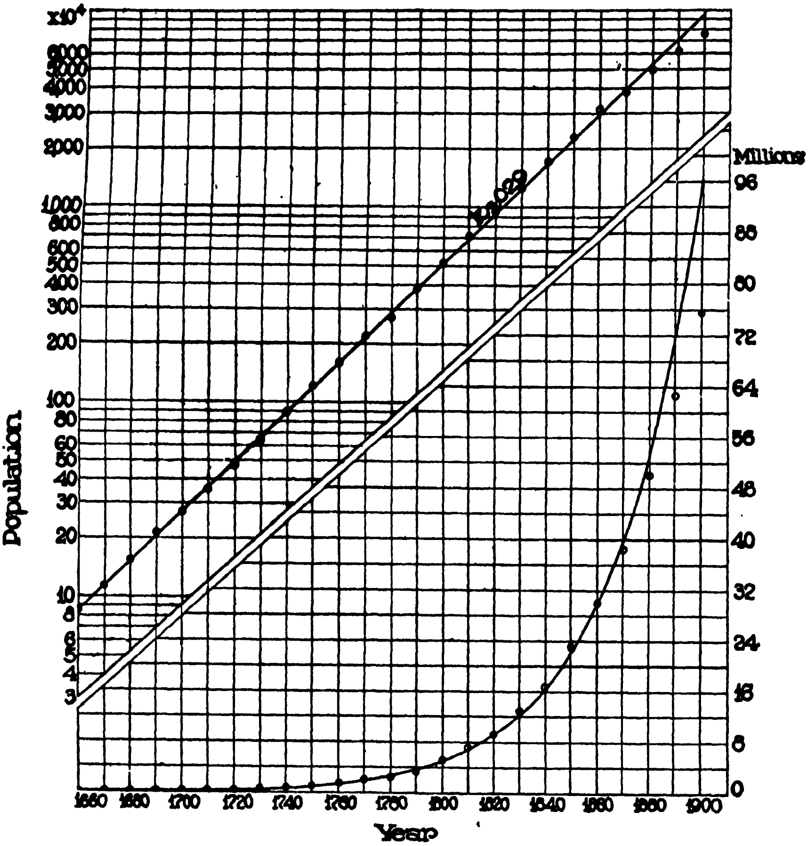

FIG. 13. The course of growth of the human population in the American Colonies and in the United States. The percentage rate is constant from 1660 to 1870. From 1870 on, the percentage rate declines in a manner indicated by the preceding figure on the growth of bacteria. $k = .029$; the population increased 2.9 per cent per year, or 29 per cent per decaded; or it doubled itself once in .693/.029 = 24 years. (Plotted from data by Rossiter, W. S., A century of population growth in the United States Bureau of the Census, United States Department of Commerce and Labor, Washington, 1909).

What we appear to have during the phase of growth preceding the inflection is a series of segments during each of which growth takes place at a constant percentage rate. These segments are separated by breaks, analogous to the breaks in the curves of cold blooded animals when undergoing metamorphosis. The present need is for growth data taken at shorter intervals in order to ascertain definitely the presence of breaks, and for an investigation of the *threshold mechanisms* bringing about these breaks, *if there are such.*

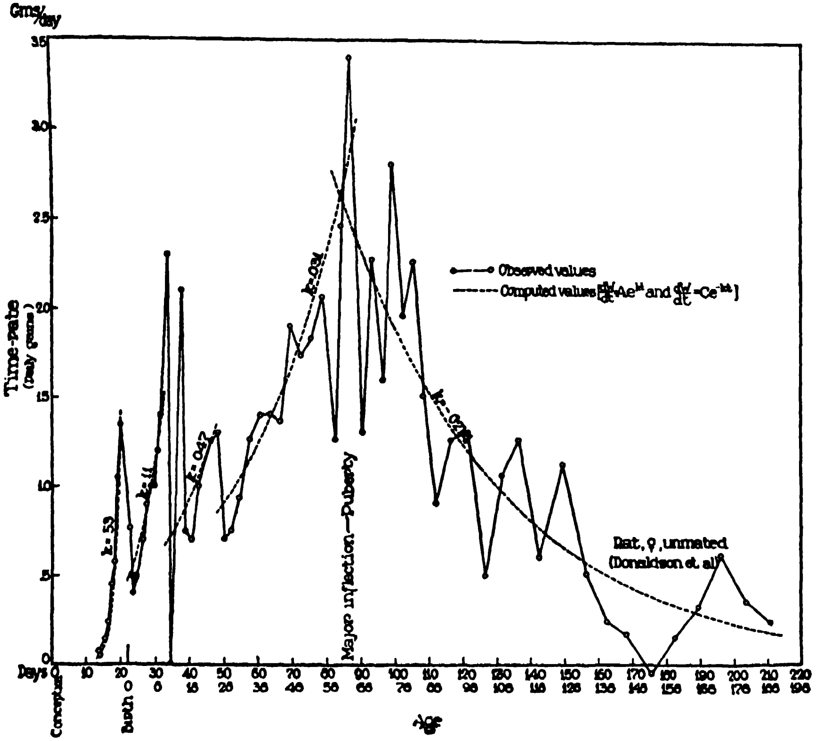

FIG. 14. The daily gains in weight of the rat plotted against age. The curve appears to have three cycles.

When the increments (time rates) are plotted against age, as shown in Fig. 14, there appear to be several cycles preceding the major inflection; as a matter of fact, the drops in the curve are not portions of cycles but breaks between successive stages of constant growth rates, as shown in Fig. 15.

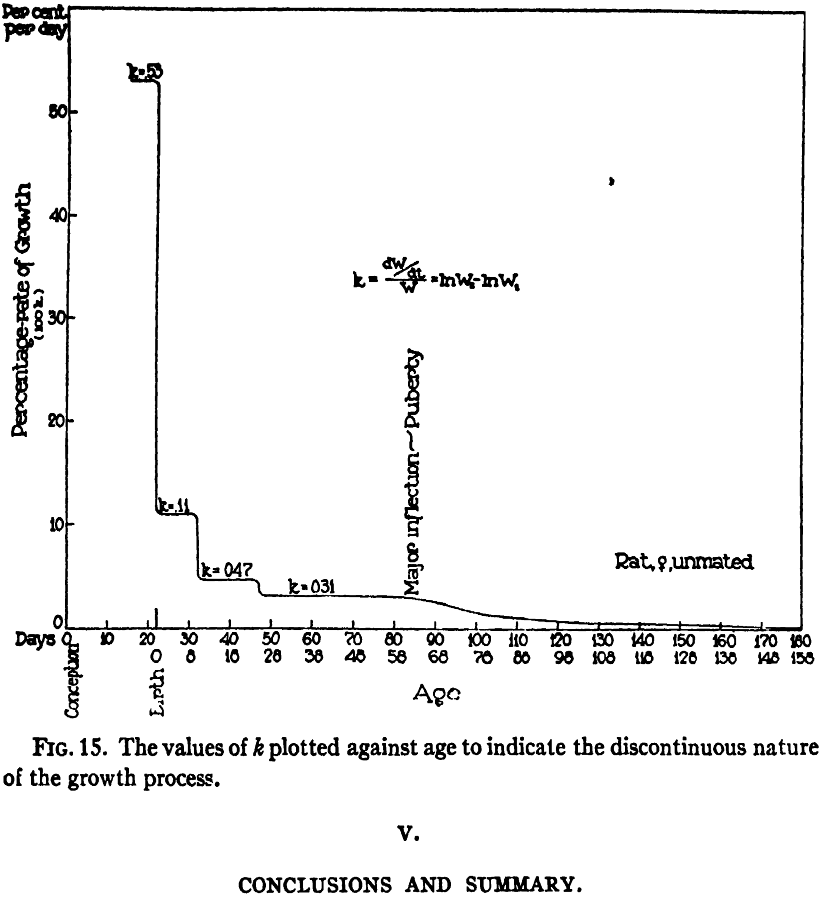

FIG. 15. The values of *k* plotted against age to indicate the discontinuous nature of the growth process.

V.

CONCLUSIONS AND SUMMARY.

Growth curves consist, in all cases, of two major segments. The first major segment is, in the case of higher animals and plants, made up in turn of several (probably five) shorter segments during each of which growth takes place at a constant percentage rate. The transitions between the successive stages are abrupt, the abruptness being of the order of metamorphosis in cold blooded animals.

It has been made clear in the first paper of this series that the time rate of growth following the major inflection *declines* at a constant percentage rate.

The junction between the two major segments occurs at puberty in animals and flowering in plants.

The two major segments are not symmetrical about the major inflection. The slope of the segment following the inflection is always less than the slope of the segment preceding the inflection. The major inflection does not occur in the center of the growth curve.

The instantaneous rate of growth at the beginning of growth is of the order of 100–200 per cent per day (*i.e.* the body weight is doubled in from 7 to 17 hours). It may be mentioned that 2 months after conception the rate of growth in man is only 8 per cent per day. This is contrary to all the published statements. Thus, Minot concluded that growth begins at 1000 per cent per day; Jackson concluded that in man, growth during the 1st month takes place at 57.5 million per cent per month; during the 2nd month 990 per cent per month; during the 3rd month 390 per cent per month (8 per cent per day is only 240 per cent per month). The reason for the discrepancy between the values derived, by the method adopted by the writer, and the values given in the literature is explained by Fig. 1.

This paper is a brief summary of Research Bulletins, 97, 98, and 99, of the University of Missouri Agricultural Experiment Station, at present in press. The reader must be referred to these bulletins for detailed discussions relating to questions that may not have been made clear in this paper.

Addendum.—Since this manuscript was submitted for publication, the writer had the privilege of discussing its subject matter with Drs. E. B. Wilson, C. R. Stockard, and H. H. Donaldson, all of whom expressed approval of the two principal ideas. Dr. Wilson called attention to a paper by G. H. Knibbs, on the Laws of population growth which appeared (on January 8) in the *Journal of the American Statistical Association*, 1926, xxi, 381, substantiating in principle one of the two principal ideas of this paper, namely that in the early history of a population the percentage rate of growth is constant. Dr. Stockard called attention to the fact that the peak in the mortality curve of the chick (Fig. 3) at 5 days is a counterpart of the peak in the prenatal mortality curve in man at 3 months. This is the junction between the embryonic period (formation of organs) and fetal period (enlargement of body and organs). The nature of the growth process in the two stages is quite different, and it is not, therefore, surprising to find a high mortality (and break in the growth curve) at this time.

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THE KINETICS OF EXOSMOSIS OF WATER FROM LIVING CELLS.

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(Accepted for publication, February 17, 1927.)

In previous communications data were presented on the rate with which water enters living cells (the unfertilized egg of *Arbacia punctulata*) under the driving force of osmotic pressure (1, 2). The present paper is concerned with the reverse process—exosmosis of water.

The material and technic of the experiments were the same as formerly employed.

The Kinetics of Exosmosis.

The first point was to determine whether exosmosis follows the same diffusion equation as does endosmosis, namely $\frac{dx}{dt} = k\,(a - x)$, where a is the total volume of water that will cross the membrane before equilibrium is established, x the amount that has already crossed at time t, and k is the velocity constant. For this purpose, eggs were placed in a dish containing 60 per cent sea water (sea water 60 parts, and distilled water, 40 parts). In this hypotonic solution, eggs were allowed to swell until osmotic equilibrium was attained. A number of eggs were then transferred to a second dish containing full strength sea water (100 per cent sea water). Three cells were measured with an ocular screw micrometer at minute intervals, until they had again reached osmotic equilibrium. Duplicate observations were usually made by the two observers. The mean volumes of 6 or more cells were plotted against times, and a curve obtained, as is shown in Fig. 1. In the same graph, $\log \frac{a}{a - x}$ is plotted against times. This plot is found to give a straight line, the slope of which is k, the velocity constant.

It is evident from this relation that the process of exosmosis follows the same equation as was previously found to fit endosmosis. This result was invariably obtained in scores of experiments at various temperatures.

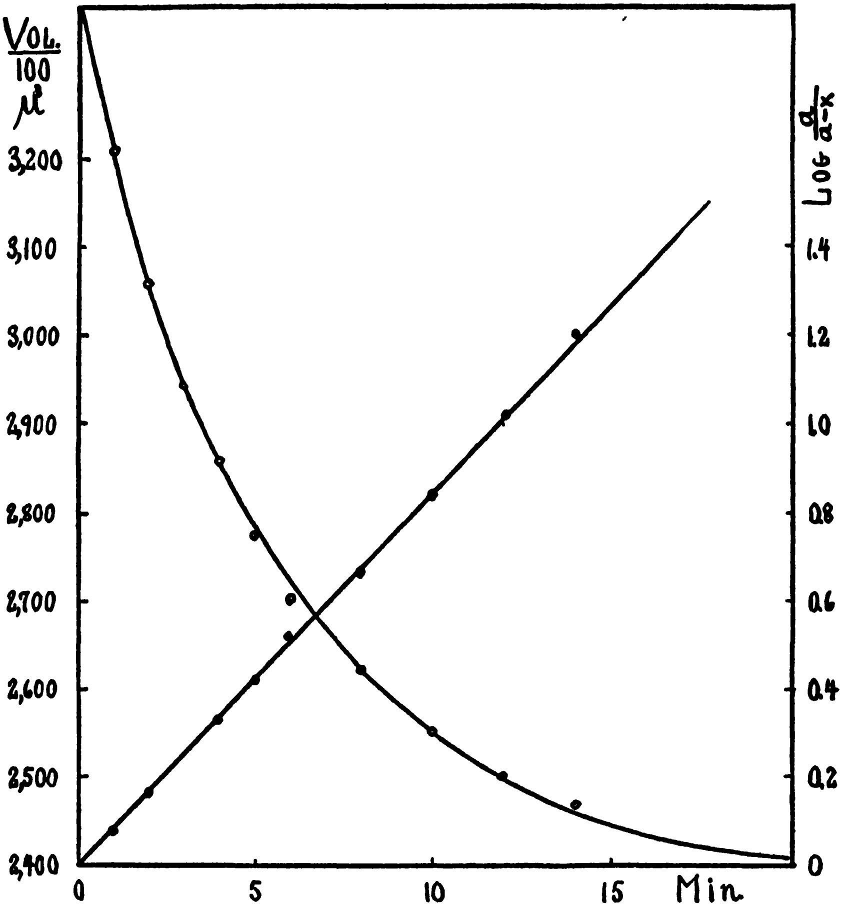

FIG. 1. A typical exosmosis experiment. Cells previously swollen in 60 per cent sea water were caused to shrink by placing them in 100 per cent sea water at 15°C. The curve represents the decrease of volume with time (open circles). The graph of $\log \frac{a}{a-x}$ against time (solid circles) is a straight line, the slope of which gives the value of k ($= 0.085$). This graph shows that the process follows the equation $kt = ln \frac{a}{a-x}$. (Each point represents the mean of 10 cells.)

Relative Rates of Exosmosis and Endosmosis.

The second point was to determine whether the *rates* of exosmosis and of endosmosis are the same. For this purpose eggs were placed in 60 per cent sea water, and velocity constants of swelling determined at several temperatures, as previously described (1). Eggs from the same animal were then returned from 60 per cent sea water to 100 per cent and the constants for the reverse process obtained. The results of a typical experiment are shown in Fig. 2, in which log $\frac{a}{a-x}$ is plotted against times, for both endosmosis and exosmosis. It is seen that at a given temperature, the velocity constant of exosmosis is greater than that of endosmosis.

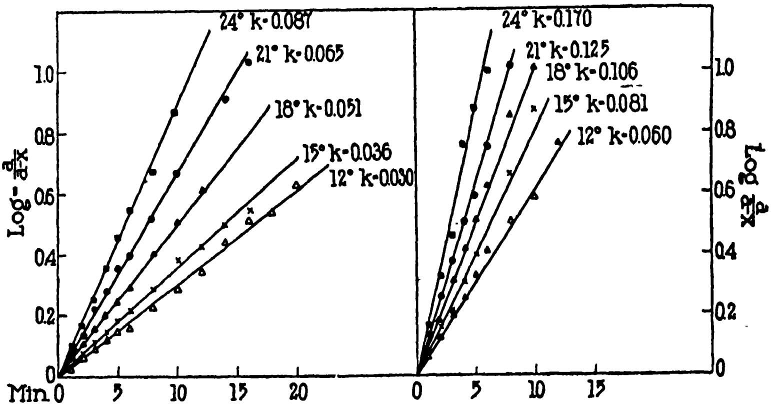

FIG. 2. The relative rates of exosmosis and endosmosis, at several temperatures. Log $\frac{a}{a-x}$ is plotted against time. The resulting graph is for each temperature a straight line, the slope of which gives the value of *k*, the velocity constant.

The graphs on the left represent *endosmosis* (swelling in 60 per cent sea water); the graphs on the right, *exosmosis* (shrinking after return to 100 per cent sea water). Under these conditions the values of *k* are, at each temperature, approximately twice as great in the latter process (contrast with Fig. 3). (Each point represents the mean of six cells.)

This experiment was repeated a number of times. While the values of *k* varied with different lots of eggs, essentially similar results were practically always obtained: the eggs shrank faster than they had swollen.

Subsequent experiments, however, showed that this difference in rate between exosmosis and endosmosis was not an essential one, that it existed only under special conditions. In the experiments just reported 60 per cent sea water was used to produce swelling of cells, but another concentration—100 per cent—to produce shrinking. Might this fact, rather than essential dissimilarities between endosmosis and exosmosis, be responsible for the differences in velocity constants? If swelling and shrinking could be produced in solutions of the *same* concentration, it seemed possible that equal values of k would be obtained.

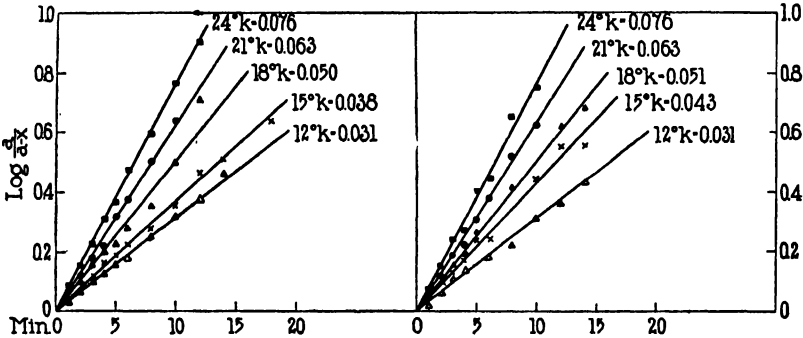

FIG. 3. The relative rates of exosmosis and endosmosis, at several temperatures, in media of the *same* osmotic pressure. The graph on the left represents *endosmosis* (swelling in 60 per cent sea water); the graph on the right *exosmosis* (shrinking in 60 per cent sea water after previous swelling in 50 per cent sea water). The medium is therefore of the same concentration in both processes; under these conditions the values of k are almost identical in exosmosis and endosmosis at each temperature (contrast with Fig. 2). (Each point represents the mean of six cells.)

Accordingly, eggs were placed in 60 per cent sea water at various temperatures and the several velocity constants determined for swelling. Other eggs were placed in a *more* hypotonic solution—50 per cent sea water. After having attained constant volume they were transferred to a 60 per cent solution and allowed to shrink, values of k being obtained as before. The results of this experiment are shown in Fig. 3. It is seen that the slope of the lines is the same for shrinking as for swelling, indicating that the velocity constants for the two processes, at each temperature, are the same.

Similar results were obtained where cells were allowed to swell in 70 or 80 per cent sea water, and other cells were allowed to shrink in solutions of the same concentration. In every experiment, practically identical values of k were obtained for swelling and shrinking at a given temperature, providing that the concentration of the medium was the same.

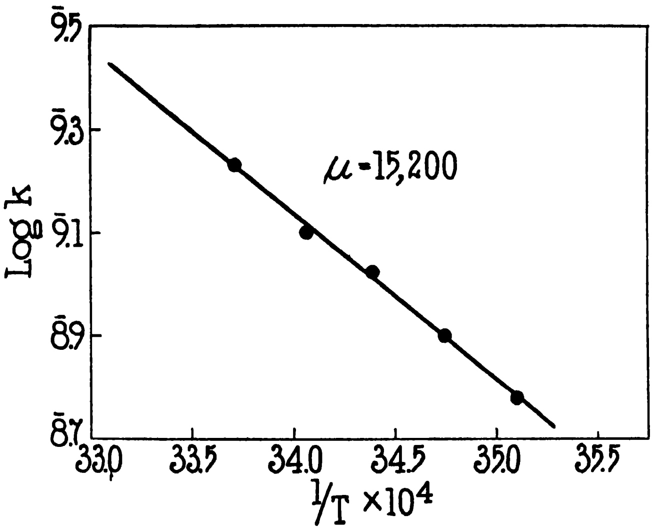

FIG. 4. The effect of temperature on exosmosis. The logarithms of the velocity constants of the experiment shown in Fig. 2 (graphs on the right) are plotted against the reciprocals of the absolute temperature. The slope of the line gives the value of μ (= 15,200).

Effect of Temperature on Exosmosis.

It follows from this as a corollary that exosmosis is affected by temperature just as is endosmosis (1), that is, there is a marked increase in rate with rise in temperature. Fig. 4 shows that the Arrhenius equation holds, the value of the temperature characteristic in this case being 15,200 (data were taken from Fig. 3). The value of μ varied in different experiments much as in endosmosis, but was always high.

These experiments therefore appear to indicate that the kinetics of exosmosis and endosmosis of water in this material are identical. The only difference in the processes is that the direction of the driving force of osmotic pressure is reversed. Both processes are affected in the same way by the external factors of temperature and concentration of sea water.

SUMMARY.

1. The rate of exosmosis of water was studied in unfertilized *Arbacia* eggs, in order to bring out possible differences between the kinetics of exosmosis and endosmosis.

2. Exosmosis, like endosmosis, is found to follow the equation $\frac{dx}{dt} = k\,(a - x)$, in which a is the total volume of water that will leave the cell before osmotic equilibrium is attained, x is the volume that has already left the cell at time t, and k is the velocity constant.

3. The velocity constants of the two processes are equal, provided the salt concentration of the medium is the same.

4. The temperature characteristic of exosmosis, as of endomosis, is high.

5. It is concluded that the kinetics of exosmosis and endosmosis of water in these cells are identical, the only difference in the processes being in the direction of the driving force of osmotic pressure.

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THE EFFECT OF SALT CONCENTRATION OF THE MEDIUM ON THE RATE OF OSMOSIS OF WATER THROUGH THE MEMBRANE OF LIVING CELLS.

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(Accepted for publication, February 17, 1927.)

The present paper reports a further investigation of the kinetics of osmosis, using the unfertilized egg of the sea urchin *Arbacia punctulata* as an osmometer. In a previous communication dealing chiefly with the affect of temperature on osmosis, it was pointed out that the rate of osmosis is influenced by a second external factor, the salt concentration of the medium (1). It is with this factor that the present investigation is concerned. The technic used was the same as that previously described.

In order to understand the mechanism involved it was desirable to study the effect of salt concentration of the medium over a wide range —from 20 per cent to 125 per cent of sea water.[1] Hence it was obviously necessary that for low concentrations endosmosis should be studied, and for high concentrations exosmosis. This procedure was justified since both processes follow the same diffusion equation,[2] and since their velocity constants are practically identical when the concentration of the medium is the same in the two cases (2).

Experiments.

One preliminary question needed to be answered. Suppose different lots of eggs (from the same animal) were placed in 60, 70, 80 and 100 per cent sea water

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[1] Sea water evaporated so that the concentration of salts is 5/4 that in 100 per cent sea water; the original pH of this solution was restored with CO_2.

[2] $\frac{dx}{dt} = k\,(a - x)$, where a is the total volume of water that will cross the membrane before equilibrium is established, x the amount that has already crossed at time t, and k is the velocity constant.

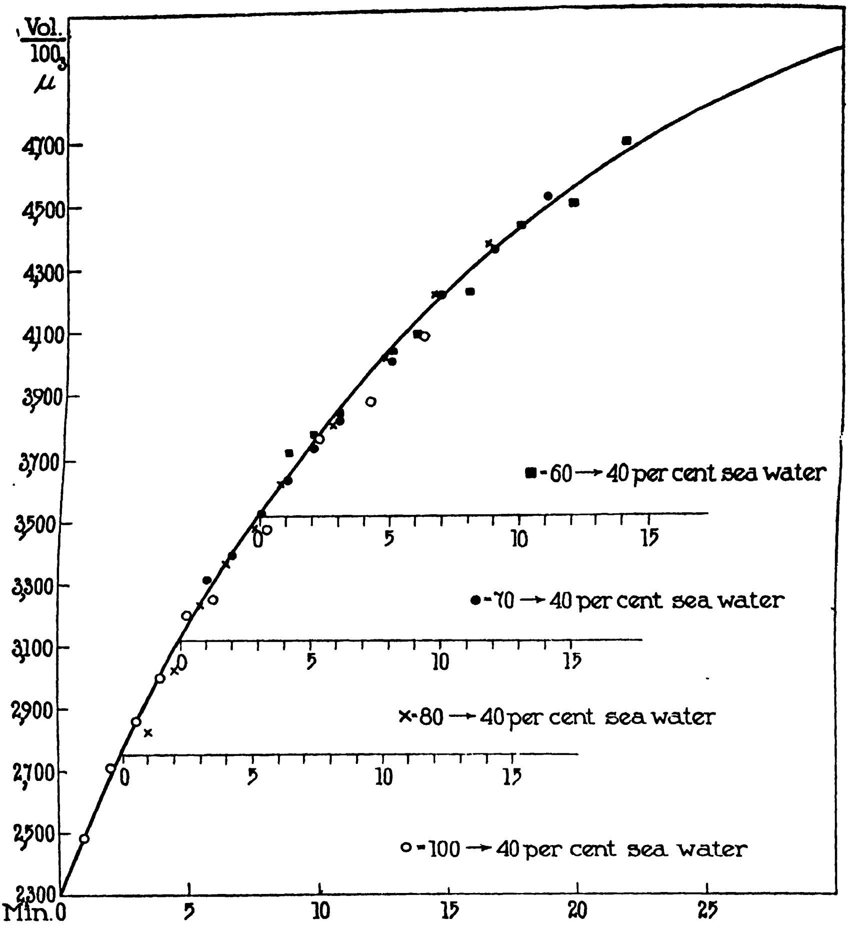

FIG. 1. Different lots of cells were placed in 60, 70 and 80 per cent sea water and allowed to reach osmotic equilibrium; then each lot was transferred to 40 per cent sea water, in which further swelling occurred. In each case the observational points from the several experiments fall along a single curve calculated from the equation $kt = \log \frac{a}{a - x}$. (In this equation the value of a was calculated as previously described (1); the value of $k = 0.027$ was determined by transferring cells from full strength—100 per cent—sea water to 40 per cent sea water.)

The points of intersection of the several base lines with the curve were found by observing the mean volume at equilibrium of twenty cells in 80, 70 and 60 per cent sea water, respectively; these values correspond to volumes at time zero in the several experiments.

The values of k in the several experiments were practically the same as the value of k for the curve. (Each observational point represents the mean of six cells.)

and allowed to reach osmotic equilibrium, and then each lot was transferred to a more hypotonic solution, *e.g.* 40 per cent sea water, in which further swelling would occur, would the values of the velocity constants be the same in all lots,—*i.e.* would volume increase go half way to equilibrium in the same length of time? Several such experiments were made, and an affirmative answer was obtained. Thus the values of *k* for swelling, on transferring the cells from the several solutions into 40 per cent sea water at 21°C., were as follows: from 100 per cent, 0.027; from 80 per cent, 0.028; from 70 per cent, 0.028; from 60 per cent, 0.026. The differences are not regarded as significant. The results are plotted in Fig. 1. In each case the observed points from the several experiments fall along a *single* curve calculated from the equation $kt = \log \frac{a}{a - x}$, as described in the legend. It is seen that the curves for cells taken from 60, 70 and 80 per cent sea water are merely upper segments of the curve for cells taken from 100 per cent sea water.

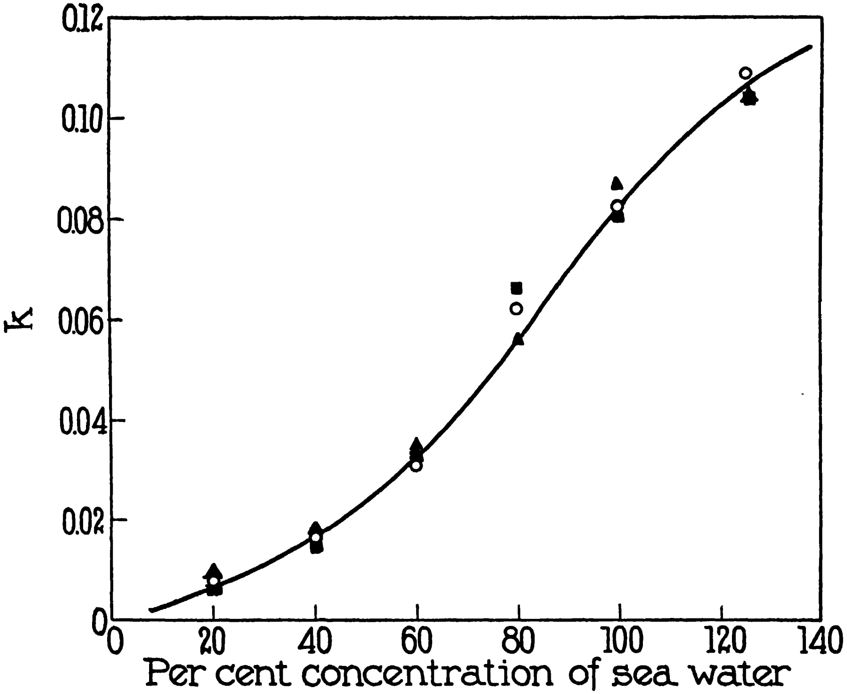

FIG. 2. The effect of osmotic pressure of the medium on the rate of osmosis of water through the membrane of living unfertilized *Arbacia* eggs. The values of *k*, the velocity constant, of three separate experiments represented by different symbols are plotted against the concentration, in per cent, of sea water. A sigmoid curve is thus obtained. (Each point represents the mean of six cells.)

Similar results were obtained on causing cells to *shrink* on being transferred from various solutions into 100 per cent sea water at 19°C., *i.e.* the values of the velocity constants were practically the same, though more variation was encountered. The results were as follows: from 40 per cent, $k = 0.15$;[3] from 50 per cent, 0.13; from 60 per cent, 0.14; from 70 per cent, 0.12; from 80 per cent, 0.15.

This point established, the problem was greatly simplified. We were justified in causing cells to swell or shrink in any convenient concentration of sea water, and then transferring them to any other concentration, the effect of which we wished to study.

We were therefore able to carry out the following experiments on the effect of concentration: cells were transferred from 100 per cent sea water into 20, 40 and 60 per cent sea water respectively. The effect of 80 per cent sea water was studied by first causing cells to shrink in 125 per cent, then to swell in 80 per cent. The effects of 100 and 125 per cent sea water were determined by first allowing cells to swell in 50 per cent and then transferring them to 100 and 125 per cent.

A large number of experiments were done of which three, represented in Fig. 2, covered the entire range of concentrations. When the velocity constants are plotted against concentration of sea water in per cent, a sigmoid curve is obtained. It is seen that the value of the velocity constant in 125 per cent sea water is more than ten times as great as in 20 per cent, at the same temperature.

These experiments were carried out at 15°C. Other experiments at different temperatures (from 12° to 24°C.), covering parts of the concentration range, also give an *S* curve at all the temperatures employed.

DISCUSSION.

The experiments show that the velocity constant of the diffusion process is altered by change in the concentration of the medium. As the medium is made more dilute (by the addition of distilled water), the velocity constant becomes smaller, and, conversely, increase in the concentration of the medium leads to increase in the velocity constant (Fig. 2). At any concentration, the rate of the process still obeys the fundamental diffusion equation, $kt = \ln \frac{a}{a - x}$. But the fact that the value of *k* depends on the concentration of the medium, points to another factor modifying the rate of diffusion. This factor would seem to be a change in permeability of the membrane to water. It appears that dilution of the medium decreases permeability to water, while concentration increases it.

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[3] The eggs had not reached equilibrium in 40 per cent sea water.

The mechanism involved may be pictured as follows:

Let us assume that water diffuses into and out of the cell through pores in the membrane, and that the size of these pores varies with the concentration of the medium, in the sense that they become larger the greater the concentration of the medium. Such a condition might be explained by regarding the cell membrane as a partially hydrated gel, which takes up water from *hypotonic* solutions, and gives off water in *hypertonic* solutions. In the one case, the gel can be imagined to swell and so reduce the size of its pores, in the other case to shrink so that the pores become more widely opened, and thus allow more rapid diffusion of water. The membrane can be thought of as permeated by the outside solution and instantly coming into osmotic equilibrium with it.

The *S* shape of the concentration effect can now be explained by the further assumption that the hydrophilic membrane takes up water at first to a great extent as the concentration of the outside sea water is decreased, but then to a less and less extent as the sea water is further diluted and the membrane approaches the saturation point. Conversely, the same reasoning can be applied when the concentration of sea water is increased—at first the membrane yields its water readily but at greater concentration only to a small extent.

This hypothesis therefore states that the lower the osmotic pressure of the sea water, the less permeable is the membrane to substances passing through its pores, and conversely. Such a theory is apparently capable of being tested experimentally.

SUMMARY.

1. Using the unfertilized egg of the sea urchin, *Arbacia*, as osmometer, it was found that the rate with which water enters or leaves the cell depends on the osmotic pressure of the medium: the velocity constant of the diffusion process is higher when the cell is in concentrated sea water, and lower when the sea water medium is diluted with dis-

tilled water. Differences of more than tenfold in the value of the velocity constant were obtained in this way. When velocity constants are plotted against concentration of medium, a sigmoid curve is obtained.

2. These results are believed to indicate that cells are more permeable to water when the osmotic pressure of the medium is high than when it is low. This relation would be accounted for if water should diffuse through pores in a partially hydrated gel, constituting the cell membrane. In a medium of high osmotic pressure, the gel is conceived to give up water, to shrink, and therefore to allow widening of its pores with more ready diffusion of water through them. Conversely, in solutions of lower osmotic pressure, the gel would take up water and its pores become narrow.

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STUDIES ON THE PERMEABILITY OF MEMBRANES.

II. DETERMINATION OF IONIC TRANSFER NUMBERS IN MEMBRANES FROM CONCENTRATION CHAINS.

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(Accepted for publication, February 3, 1927.)

1. THEORY.

It has been shown (1) that the P.D. of a concentration chain, *e.g.* with two KCl solutions in the ratio 10:1, across a membrane of dried collodion, reaches its maximum value better the more dilute the KCl solutions are, and that in higher ranges of concentrations, the effect of the membrane vanishes more and more. So in the latter case the P.D. approaches closely that established without a membrane. This is, in the case of KCl, practically equal to zero.

The interpretation of the effect of the membrane was given as follows: Any potential difference between two aqueous solutions of different electrolytic content, in direct contact with each other, is due to the difference in the mobilities of the different kinds of ions. In the ultracapillary spaces of the membrane, the mobilities of the ions are not only changed with respect to their absolute value, but also to their relative value. In general, the mobility of the anion is considerably more decreased than the cation.

Let us consider a very thin layer of the liquid content within a pore channel confined by two cross-sections through the channel, infinitely close to each other. Let the two sides of the membrane be in contact with two different solutions of the same kind of electrolyte consisting of two univalent ions. A transition zone will be formed across the membrane. Then the P.D. between two infinitely close neighboring cross-sections through this transition zone will be:

$$dE = \frac{RT}{F}\,\frac{u - v}{u + v}\,\frac{dc}{c} \tag{1}$$

where u and v are the mobilities of the cation and anion, respectively, within the concerned cross-section; c is the concentration, or better, the activity of the electrolytes within this infinitely thin layer of the liquid, and dc the change of c from the one cross-section to the neighboring one. In order to find the total P.D. from the one side of the membrane to the other, the above formula must be integrated over all the infinitely small cross-section layers from the one side of the membrane to the other. Let us designate the direction along the axis of the pore channel as the x axis; the integration has to be performed from $x = 0$ to $x = D$, where D is the total thickness of the membrane. Now, from the experiments referred to, we have to infer that u and v within the channel depend considerably on the concentration in a degree much more pronounced than in a free aqueous solution. Usually in an aqueous solution u and v depend only slightly on the concentration. Here, with a certain approximation, we may consider u and v as constant, and the result of the integration becomes:

$$E = \frac{RT}{F} \cdot \frac{u - v}{u + v} \, ln \, \frac{C_2}{C_1} \tag{2}$$

when C_2 and C_1 are the two concentrations of the solution which are in contact with each other and connected by a transition zone. It should be emphasized that this result of the integration does not depend on the manner in which the transition of the one concentration to the other is realized. It does not depend on whether the fall of the concentration is rectilinear or arranged in some other way; whether the transition is realized by a pure diffusion zone or by a zone of mechanical mixtures of the two solutions.

However, within the membrane there is no constancy of u and v over the range of integration, u and v depend strongly on c, and, therefore, also on x. In the integration, the factor containing u and v must not be placed before the integration sign, and the result is:

$$E = \frac{RT}{F} \int_{x=0}^{x=D} \frac{u - v}{u + v} \, dc \tag{3}$$

If, instead of this accurate formula, we apply formula (2), the letters u and v no longer have the meaning of the mobility of the cation and anion, respectively, but they may be interpreted as some kind of average mobility,[1] within the range of concentration C_1 and C_2. Such an average mobility has no simple physical meaning. It need not be the arithmetical mean.

In the ordinary concentration chain without a membrane, we are allowed to calculate the relative value of u and v, or the transfer numbers $t^+ = \frac{u}{u+v}$ and $t^- = \frac{v}{u+v}$ from the measurement of the P.D. availing ourselves of the formula (2) which solved for t^- reads, for a temperature of 20°C.:

$$t^- = 0.5 - \frac{E}{2 \times 58 \times \log^{10} \frac{C_2}{C_1}} \tag{4}$$

where E is the measured P.D. in millivolts.

If we apply this formula to a concentration chain with a membrane, the obtained transfer number of the anion, t^-, is nothing but the calculated average value of t^-, which has not a simple physical meaning. However, when the ratio $C_2:C_1$ is small enough, the variation of t^- for the concentration C_2 and C_1 may be also small, so that with a certain approximation the calculated t^- may be regarded as a useful approximation to both the value of t^- for C_2 and for C_1. Using this idea, there is obtained a method for calculating, with a certain approximation, the transfer number of the ions of an electrolyte within the pores of the membrane for any concentration of the aqueous solution in contact with the membrane and in equilibrium with the electrolytic content of the membrane pores.

The method consists in the measurement of the P.D. of concentration chains with a membrane, the ratio of concentrations of the two solutions being as small as possible. It is not practical to apply very small ratios, for in this case the measured P.D. becomes very small also and the inevitable error of, let us say 0.5 millivolt, which may occur in the measurement of such a chain and which may be due to uncertain and irreproducible liquid junction potentials, has too high an influence on the results. The best ratio of concentrations seemed to be the one of 2:1. The maximum effect, when the mobility of the anion = 0 is in this case 17.6 millivolts for 20°C.[2]

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[1] It should be borne in mind that according to the interpretation given in the previous paper, even the mobility of an ion, especially of an anion, within *one* very thin cross-section layer of a channel, is but an average value, as the anions fixed at the wall and the free anions in the center of the pore have different mobilities. The average value of these mobilities is called here the mobility of that ion within this cross-section. In this present paper the "average" mobility of an ion means the average of the thus defined mobilities, over the range of all of the cross-sections through the channel from its one opening to the other.

Several previous authors emphasized the fact that the transfer numbers are altered in membranes. As all of these authors applied methods different from those used in this paper, and as review of these findings is to be made in a subsequent paper, we refrain here from dis-discussing the literature, in order to avoid repetition, especially because the essential point,—the rapid change of the transfer number according to the concentration, has not yet been described so far as we know.

2. *Technique.*

The experiments were carried out with the dried collodion bag membrane, as described in the previous paper. Some series were made with flat membranes which have advantages to a certain extent; particularly in that they maintain their original properties as measured by the *Co P* virtually permanently. Such flat membranes were used by the authors through many successive experiments over months, the *Co P* having remained constant within some tenths of a millivolt. Flat collodion membranes have been used in the past, *e.g.* by Bethe and Toropoff (2), Bigelow and Gemberling (3), Bartell and Carpenter (4), and Hitchcock (5), and they have been used also long ago for ultrafilters by Bechhold, Zsigmondy, and others. But these authors worked with more or less well permeable, not completely dried collodion membranes. It is very easy to obtain this kind of membrane in a flat form, but it requires special technique to make completely dried membranes in a flat form. Recently Collander (6) described the flat form for the dried collodion membrane emphasizing its great stability. The difficulty consists in the fact that the flat membranes, while drying, shrink and shrivel and become inelastic and rigid, so

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[2] The error involved in the fact that a concentration ratio is used instead of one between activities, is so small for a ratio of 1:2 as to be negligible with respect to other errors involved.

CORRECTION.

On page 675, Vol. X, No. 5, May 20, 1926, fifth line from the top, *75 per cent ether and 25 per cent alcohol* should read *75 per cent alcohol and 25 per cent ether.*

that they cannot be fastened without a leak to any opening of a vessel. One has to fix the membrane, in a half dried condition, to the rim of a vessel and then allow it to dry. The authors found the following method best.

A 5 per cent solution of celloidin Schering in 75 per cent ether and 25 per cent alcohol was poured on a mercury surface in a Petri dish, as Bartell suggested, in an amount sufficient to cover easily the whole surface by the spontaneous spreading of the viscous solution. The collodion is allowed to dry to such an extent that the consistency is just suitable for cutting with a knife. At this time, the periphery adjacent to the rim of the Petri dish is cut and the membrane taken out and put over the opening of a bell jar shaped glass, such as is shown in Fig. 1. The diameter is 2 cm. smaller than that of the Petri dish in a cross section. The rim is represented by an even ring 1 cm. broad with ground surface. The rim is wet with a layer of collodion solution immediately before the half dried membrane is put on. By means of this the membrane is glued on the rim. While the drying process is going on, the main part of the membrane covering the opening of the jar is prevented from shrinking. However, the peripheral portion of the membrane shrinks in such a way that a circular sulcus outside the glass rim is formed, the concavity being directed toward the pointed part of the jar. Now the jar is turned over and collodion solution is poured into this sulcus. This fills in the leaks which may be left between the membrane and the glass rim. Moreover, while drying and shrinking, the peripheral collodion ring shrinks and rolls so that finally a tight band hermetically seals all leaks. The membrane has to stand 3 or 4 more days to complete dryness which can be recognized by electrification after rubbing with hair. The membrane is exposed to an enormous

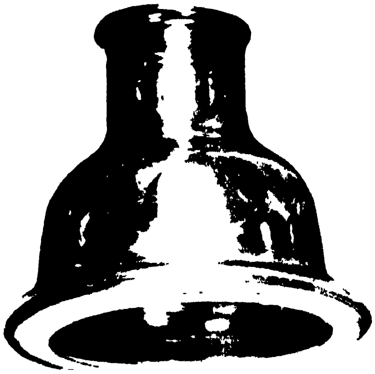

FIG. 1. Glass bell jar used as supporting frame for flat membranes.

stress, and too thin a membrane sometimes will crack. Those membranes which have withstood the stress have turned out to be virtually invariable in any respect. The *Co P*, even after months of experiments, remains constant within a fraction of a millivolt. It is true, in consequence of the complete lack of shrinking, the *Co P* does not reach such high values as sometimes is obtained in the shrunk bag membranes. Usually a *Co P* of about 48 millivolts was obtained, whereas the other form sometimes gives 52 to 53 millivolts. In the second place, on account of the thickness, a long time is necessary to reach a stationary condition when measuring the P.D. To measure, for example, the *Co P*, the membrane has to stand at least some hours in contact with the solution, the latter being several times renewed. However, after the stationary condition has been established, the P. D. keeps astonishingly constant for any period.

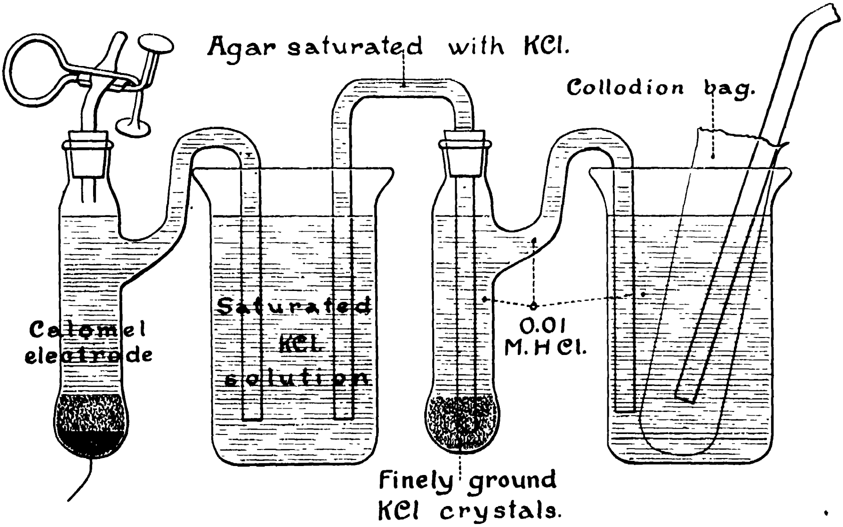

FIG. 2. Method of connecting calomel half-cell with membrane system to avoid diffusion potentials and diffusion.

When a membrane used for one experiment is to be used for another with different solutions, the previous solutions must be washed out at least 24 hours; and in certain cases, *e.g.* in the transition of KCl chain to a LiCl, where any trace of the much more movable K will spoil the value of the Li chain, the washing process should be prolonged several days. Inconvenient as that may be, the labor is rewarded by very constant and reproducible results, and on account of its invariability, such a membrane can be used for comparative successive experiments with different solutions much better than the bag membrane.

As an error of even 1 millivolt has a relatively great influence on the calculation of the transfer numbers, the errors involved in liquid junction potential should be eliminated as far as possible. In chains with KCl there is no trouble in this respect, but for other electrolytes the arrangement shown in Fig. 2 was used. This represents a .01 M HCl solution outside the membrane connected with the leading off calomel half cell. The same arrangement was used to connect the inside solution of the collodion bag with the other electrode. This arrangement is based on the principle of Michaelis and Fujita (7), interposing between the HCl solution and the saturated KCl solution another HCl solution of the same concentration as the first one, but also saturated with KCl. We may be sure that the liquid junction potential is reduced to a fraction of 1 millivolt at least. The two calomel half cells, saturated with KCl, were often checked against each other and when a small P.D. was observed between them, a suitable correction for the P.D. of the chains in the experiments was made.

3. Results and Discussion.

Table I shows the P.D. of a chain with a dried collodion bag membrane between two KCl solutions of the concentration 1:2, in different ranges of concentration. The last column gives the transfer number for Cl evaluated according to formula (4) (Fig. 3, lower curve).

Table II shows a similar experiment with a flat membrane. Here the constancy of all values is best, though long time and repeated change of the solution was necessary to reach these constant potentials. The definite value of this series is most trustworthy of all, and the smoothness of the interpolation curve (Fig. 4) confirms this assumption.

Table III gives a series of experiments with a fairly stable bag membrane and with the chlorides of H, K, Na, and Li. The entire series would have consumed too much time, had it been made with a flat membrane. So the results may not be quite so accurate in an average, though much care was taken to obtain a really stationary condition by extended observation and repeated renewal of the solution before definite readings were made. The results are plotted in Fig. 5.

Although the transfer number of Cl is low in any case, especially in low concentration ranges, still the difference can be plainly seen in the different alkali chlorides. The experiments with HCl were not carried out up to the same concentration as in the other electrolytes on account of doubt as to whether liquid junction potentials might be insufficiently abolished. It should be borne in mind that an error of 1 millivolt, according to the particular circumstances, may have a great influence on the evaluated transfer number. That is also the reason why these figures are not utilized in further computations but only shown in the diagram where the interpolation curves, obtained a little arbitrarily, give an idea about the differences. The order of the transfer number of Cl in the different chlorides is the same as in aqueous solutions; the absolute values, however, strongly diminished in comparison with the latter, and the fact that the transfer number depends largely on the concentration, is obvious for all of these electrolytes, whereas in free aqueous solutions the influence of concentration is known to be small. Further discussion must be delayed.

TABLE I.

Celloidin Membrane, Bag Form.

Change of the P.D. *with the Change of the Concentration Range. The Ratio of Concentration of Each Chain is Always 1:2. All Experiments with the Same Membrane.*

KCl solutions	P. D.				
	1st series of experiments (22°C.)	2nd series (22°C.)	3rd series (20.5–22°C.)	Average	t_{Cl}
	mv.	*mv.*	*mv.*	*mv.*	*mv.*
M/400 : M/200		17.8	17.7	17.7	0
M/200 : M/100	15.3		17.8	16.6	0.040
M/100 : M/50	14.7		16.2	15.5	0.059
M/50 : M/25			15.3	15.3	0.065
M/25 : M/125			12.5	12.5	0.145
M/20 : M/10	13.2	13.0	12.9	13.0	0.130
M/12.5 : M/6.25			9.5	9.5	0.230
M/6.25 : M/3.125			5.5	5.5	0.309
M/2 : M/1	4.8	3.9	3.8–3.1 (Aver. = 3.4)	4.0	0.386

Theoretical maximum value at 22° = 17.6 millivolts.

TABLE II.

Celloidin Membrane, Flat Form.

Concentrations of KCl	Temperature	P. D.	t^{-}_{Cl}
		mv.	
N/1 : N/2	18.5°	3.5	.399
N/2 : N/4	18.5°	5.45	.343
N/4 : N/8	19.5°	9.55	.226
N/8 : N/16	19.5°	11.75	.163
N/16 : N/32	19.5°	14.5	.085
N/32 : N/64	20.5°	15.4	.060
N/64 : N/128	20.5°	16.35	.033

TABLE III.

Celloidin Membrane, Bag Form.

Temperature 20° ± 0.5°C. Theoretical Maximum Value of a Concentration Chain for This Temperature: 17.4 Millivolts.

	Concentration range	P. D.	Transfer Number of Cl
	M	*mv.*	
LiCl	.01–.02	15.1	.066
	.02–.04	13.4	.115
	.04–.08	11.4	.173
	.08–.16	8.3	.262
	.16–.32	5.2	.351
NaCl	.01–.02	15.5	.055
	.02–.04	14.8	.075
	.04–.08	13.5	.112
	.08–.16	10.5	.198
	.16–.32	6.2	.322
KCl	.01–.02	16.05	.026
	.02–.04	15.4	.058
	.04–.08	13.6	.109
	.08–.16	10.8	.190
	.16–.32	7.3	.290
HCl	.01–.02	17.2	.006
	.02–.04	17.1	.009
	.04–.08	15.65	.050
	.08–.16	15.5	.055

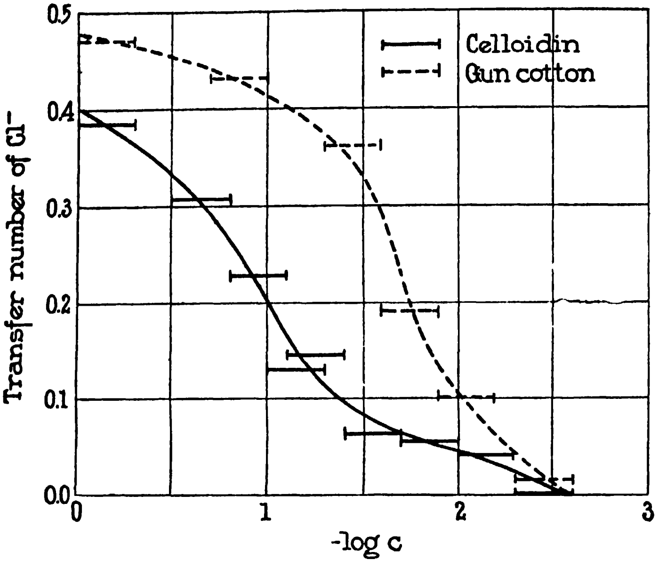

FIG. 3. Magnitude of the transfer number for Cl through the different concentration ranges with two membranes of the bag type.

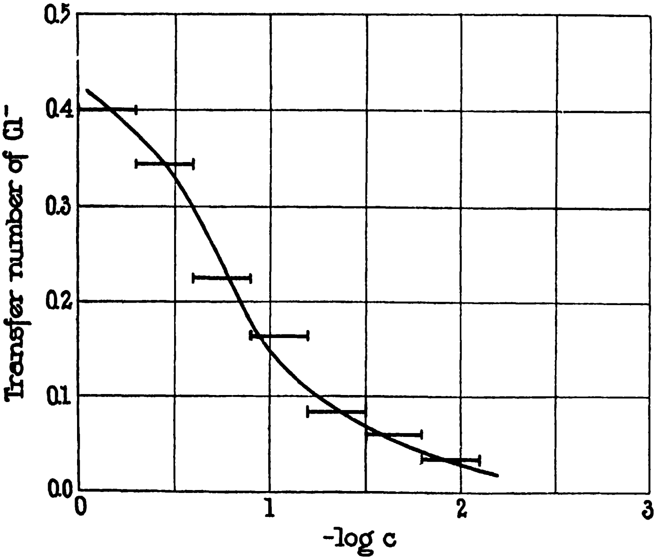

FIG. 4. Magnitude of the transfer number for Cl through the different concentration ranges with a collodion membrane of the flat bell jar type.

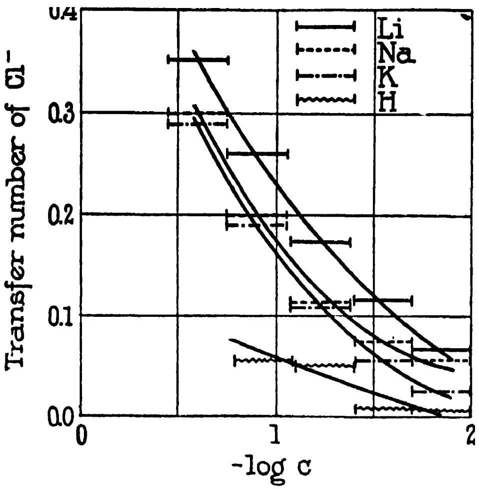

FIG. 5. Concentration chain curves showing the transfer numbers for Cl in the form of chlorides of different cations.

TABLE IV.

Gun Cotton Membrane, Bag Form.

Temperature 26.5°C. Maximum Value Calculated 17.7 Millivolts

KCl solutions	P. D.	$t_{\overline{\mathrm{Cl}}}$ calculated
	mv.	
M/1 : M/2	1.10	0.47
M/5 : M/10	2.60	0.43
M/10 : M/20	4.05	0.40
M/20 : M/40	5.55	0.36
M/40 : M/80	10.90	0.19
M/80 : M/160	14.25	0.10
M/160: M/320	17.1	0.017
M/10 : M/100 (that is : *Co P*)	21.4*	0.306†

* 2 days ago 23.0.

† Theoretical maximum value for this chain = 55 millivolts. Corresponding to the value of the interpolation curve for a concentration = M/40.

In order to show that this behavior of the membrane is connected with their relatively high *Co P*, being only a couple of millivolts below the possible maximum value, a gun cotton membrane with a lower *Co P* (21 to 23 millivolts) was used for a similar series with KCl (Table IV and Fig. 3, upper curve). It can be seen that the transfer number of Cl is always greater than the one with a good celloidin membrane (lower curve, Fig. 3) through the same concentration range. However, in principle, the course of the curve is the same.

It should be borne in mind that the calculation of the transfer numbers in this paper are completely based on the assumption that the P.D. of the concentration chains with the membrane are due to the difference of the mobilities of the cation and the anion. This assumption was till now only an attempt to control the phenomena in membranes by a simple hypothesis, and the present paper shows some consequences of this hypothesis. Subsequent papers will have to deal with the problem of whether this hypothesis is sufficient or requires an addition or correction.

SUMMARY.

The ionic transfer number in an electrolyte solution in the pores of a narrow pored collodion membrane depends much more on the concentration than it does in a free aqueous solution. The potential difference of two solutions of the same electrolyte in different concentration depends largely on the concentration range. The ratio of the concentrations on the two sides was always 1:2 in the experiments; the concentration range was varied. It is shown that the transfer number of Cl, calculated from the P.D. measured, is very small in dilute solution (down to .02 and less in some cases), whereas it approaches the value .5 holding for free aqueous solutions when the concentration range is raised. The differences for the transfer number of Cl, according to the cation (H, K, Na, Li), can be recognized and show the same order as in free aqueous solution. But even in LiCl, where in an ordinary aqueous solution the transfer number of Cl is always $> .5$, this number is very low in the case of the membrane (*e.g.* $< .05$ in .01 M solution).

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STUDIES ON THE PERMEABILITY OF MEMBRANES.

III. ELECTRIC TRANSFER EXPERIMENTS WITH THE DRIED COLLODION MEMBRANE.

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1. INTRODUCTION.

In a previous paper (1) an indirect method was described for determining the transfer number of an electrolyte in the dried collodion membrane. To furnish additional evidence in favor of the theory, it has seemed desirable to determine the transfer number by direct transfer experiments with an electric current. For a long time it has been known that the transfer number might be altered by a membrane. A number of investigators had tried to make use of various types of membranes to separate the different regions of the transfer apparatus and thereby avoid mechanical mixture. But as soon as it was recognized that the transfer number might be changed by the membrane, the method was abandoned. In the present investigations, however, this change of the transfer number is the very object of the investigations.

The alteration of transfer numbers by membranes observed by the earlier investigators is discussed by Hittorf (2), who found that only certain membranes, in which what he called the "Schlierenphænomen" could be observed, were able to cause any change. This phenomenon can sometimes be observed when two equal solutions are separated by a membrane and an electric current passed across the membrane in such a way that electroendosmosis occurs. Normally in the phenomenon of electroendosmosis the solution as a whole is forced unchanged through the membrane in the direction of the positive current. But in some cases (especially with membranes of gelatin and dried animal intestine and with electrolytes such as $CdCl_2$, $CaCl_2$, and HCl, but never with the usual uni-univalent neutral salts) the solution was separated into two different parts by the electroendosmosis. The solution coming through the membrane in the direction of the positive current was of a lower concentration than the original solution and that going in the opposite direction of higher concentration. The two layers could be distinguished with the naked eye because of refractive differences and even better with Töpler's "Schlierenapparat" (3). According to the contention of Hittorf it was only in those membranes which gave rise to this "Schlierenphænomen" that the transfer number differed from the one determined without a membrane. When the phenomenon was absent he believed that the membrane had no influence on the transfer number. Among the membranes showing the "Schlierenphænomen" Hittorf gives brief mention to the collodion membrane. His collodion membranes were in all probability of the dried type used in the present investigations, a fact which can be recognized because of the high electrical resistance which he attributed to them. The more common permeable collodion membranes of the present day when in contact with an electrolyte solution do not offer any conspicuous resistance to the passage of a current. Unfortunately Hittorf's comments on these changes of the transfer numbers are exceedingly brief and the change produced by collodion membranes was not determined. Relatively the observed alterations were small and just great enough to justify the claim that the membrane marred somewhat the accuracy of a transfer number. They did not approach in degree the changes of transfer number by membranes of dried collodion reported in our last paper nor was any note made of dependence on concentration.

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* This investigation was begun by Dr. Yamatori while working in my laboratory in Nagoya, Japan. Due to lack of time he was unable to complete the experiments, but the Baltimore authors are greatly indebted to him for preliminary work in which the difficulties associated with these transfer experiments were recognized and overcome.

Related to the Hittorf phenomenon is the phenomenon described by Bethe and Toropoff (4) which may be regarded as a special case of the more general Hittorf phenomenon. These authors described a sort of hydrolysis brought about by a membrane. When a membrane is interposed between two equal and very dilute solutions of a neutral salt and an electric current allowed to pass, an acid reaction is brought about immediately adjacent to one side of the membrane and an alkaline on the other. This phenomenon is a special case of the Hittorf phenomenon insofar as a change of concentration of certain ions, namely H^+ and OH^-, is brought about by the membrane. This phenomenon can be observed in an appreciable degree only in salt solutions of such a high dilution that the ions of water participate appreciably in conducting the current within the membrane. As will be shown later this effect is small enough in our experiments to be neglected for the computation of the transfer numbers of the ions of the neutral salt.

The Hittorf effect is likewise closely associated with a phenomenon of electric transfer described by Nernst and Riesenfeld (5). When two equal aqueous solutions of an electrolyte are separated by a third conducting layer which contains the same electrolyte but is not miscible with water and an electric current is allowed to pass through the system, at times the electrolyte becomes concentrated on one side of the third layer and diluted on the other. Nernst and Riesenfeld discerned that this effect must be the result of a difference in the transfer number of the electrolyte in the aqueous and non-aqueous solutions and showed how the transfer numbers could be calculated from such experiments. Riesenfeld (5) made a number of these determinations, using such systems as water-phenol-water, containing electrolytes like KI or KCl in equilibrium throughout the system. The transfer numbers for the cation with KCl were as high as 0.8. He then attempted to determine these transfer numbers by another method; *i.e.*, by measuring the E. M. F. of concentration chains, but was unable to confirm the values obtained in the transfer experiments, the results of the second method giving a figure close to 0.64. A solution of these contradictory results was not attempted by Riesenfeld, nor has anyone undertaken a renewed investigation as far as we know. The membrane used by Riesenfeld differed from ours in being a homogeneous phase working as a solvent for the electrolyte as opposed to our dried collodion membrane which in all probability acts as a sieve. Nevertheless, this phenomenon of Nernst and Riesenfeld[1] observed in homogeneous membranes as well as the phenomenon of Hittorf observed in porous membranes can both be reduced to a difference of transfer numbers of the electrolyte within and without the membrane.

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[1] Only a brief discussion of this work by Nernst and Riesenfeld has been inserted here because it is not directly concerned with the properties of porous membranes. A complete review must be left for a more suitable occasion.

Our own experiments deal with the dried collodion membrane in which the determination of a transfer number is simplified in one respect though complicated in another. It is simpler because the error introduced by spontaneous diffusion, convection, and mechanical stirring is small and usually may be neglected. Separation of the different parts of the fluid is accomplished by the membrane. On the other hand a new source of error is introduced. At times the initial properties of a membrane may be markedly altered by the action of a strong electric current. These changes are partly reversible and partly irreversible; *i.e.*, permanent even after the electric current has been stopped. This action of an electric current on a membrane may be described and a method of obviating the difficulties involved pointed out.

2. *The Behavior of the Dried Collodion Membrane in the Electric Current.*

If two equal solutions of some electrolyte, *e.g.* Na_2SO_4 in 0.1 or 0.01 M concentration, are separated by a bag of completely dried collodion and a rather strong electric current (0.1–1 ampere) is allowed to pass between a platinum electrode inside and another outside the bag, after a certain time a sharp cracking noise will be heard, such as is produced by a spark breaking through an insulator. The noise soon becomes a permanent rattle and on inspection one can see a small leak which is apparent because of a rapid streaming of the liquid through it from one side of the membrane to the other. The stream is visible because of a difference of light refraction. When the leaks are of microscopic size the holes cannot be seen and are recognized only by this phenomenon of refraction. Gradually the leak becomes larger and a macroscopic hole can be seen. Simultaneously with the formation of a leak the intensity of the electric current begins to increase rapidly (unless diminished by the application of an external resistance) due, of course, to the increased conductivity through the hole in the membrane.

The description of this phenomenon as given is far from being complete, and must be studied more thoroughly. However, for the present purposes it is sufficient to point out that the electric current exerts some influence on the pores of the membranes. Only when the voltage is high will the current strength be sufficiently great to cause an actual break in the membrane. However, when the voltage is lower, the same influence may be present though not great enough to produce a rupture. This supposition is rendered likely by the observation that the *Co P* of a membrane may be much lower after than before the weak current was passed. For example, one bag membrane with a *Co P* of 50 millivolts between 0.1 and 0.01 M KCl solutions, showed a P.D. of only 30–40 millivolts after the passage of the current. As a special procedure is necessary for measuring the *Co P* of membranes after the application of an electric current, a description of this procedure will be inserted here.

When the ordinary dried collodion membrane is placed between two KCl solutions of the same concentration, there is, of course, no P.D. However, after a membrane has been subjected to the passage of an electric current this may no longer be true. A P.D. is established even between two equal solutions. Evidently some kind of polarization has taken place. The distribution of the electrolyte ions within the membrane is no longer homogeneous. One side has become more concentrated, the other more dilute, and hence the P.D. The P.D. tends to fall but the fall is sometimes very slow especially when the membrane has been washed in distilled water. Even after a day of such washing a part of the polarization potential may still remain when the membrane is placed between two 0.01 M KCl solutions. However, it can quickly be diminished by washing with a stronger salt solution instead of pure water. When after washing with water some millivolts are established between two 0.01 M KCl solutions, the P.D. between two 0.1 M KCl solutions is always much smaller. When the membrane is allowed to stand for some minutes in the stronger solution and then replaced in the weaker one, the P.D. will have been definitely diminished. In this way one can depolarize a membrane completely, so that no P.D. will be established between two 0.01 M KCl solutions. Not until this behavior of the membrane has been reached can the P.D. between two *different* KCl solutions (0.1 and 0.01 M) separated by the membrane be measured with any reasonable meaning. With any membrane which has been subjected to the passage of an electric current the absence of polarization must be strictly proven before its *Co P* is measured. When the current has been weak (<0.1 milliampere) and the time of exposure short, the polarization potential is usually small and easily overcome.

It is obvious that the permeability of a membrane will change when the pores have been dilated by the electric current. Just as the *Co P* is diminished, so will the difference in behavior between cations and anions be decreased. It is for this reason that in our first experiments we did not succeed in observing transfer numbers for the anion as low as had been expected. To be sure a transfer number of the anion with KCl close to 0.5 was never found. All experiments tended to confirm a diminished velocity of the anion in the membrane but the diminution was not great enough and not regular enough in the different experiments. When the difficulties had finally been recognized, it became possible to obtain very low transfer numbers for the anion. Those experiments were selected in which the *Co P* of the membrane turned out not to have been altered by the current. To insure this it was found necessary to apply only weak currents for a short time. As a consequence the quantity of ions transferred was very small and chemical analyses of a high degree of accuracy were not possible. Nevertheless by a slight modification of the procedure, to be described presently, the results of the chemical analyses were in excellent agreement and entirely sufficient for the purpose even though the limits of error were somewhat higher than under more favorable conditions.

Since the development of flat membranes of the type described in a previous paper (1), the difficulties due to alteration of the membrane by the current have been largely abolished. The properties of these membranes, as estimated by the *Co P*, have remained unchanged to the present time, a period of more than 4 months, even though used almost daily for transfer experiments. In the future there should be no difficulty in performing transfer experiments with collodion membranes.

3. Arrangements of Transfer Experiments.

The use of a dried collodion membrane to separate the different solutions in an electric transfer experiment is simple and convenient when compared with the usual transfer experiments made in electrochemistry. The most simple arrangement is to separate two equal solutions of an electrolyte by the membrane, allow the current to flow, and then determine the change of concentration in the two solutions. This method, however, could not be used because the quantity of current necessary to change the concentrations sufficiently for satisfactory chemical analysis was large and even the most resistant membranes would eventually have been injured, especially when used for a long comparative series of experiments. Fortunately it was possible to modify the procedure so as to furnish better facilities for chemical analysis. Solutions of KNO_3 and NaCl of equal molarity were separated by the membrane, platinum electrodes introduced into each solution, and a current allowed to pass in the direction from KNO_3 to NaCl. In the very beginning of the experiment, before the circuit had been closed, the membrane would contain the ions of both of these two electrolytes. However, within a few moments after the current had been started, the Na^+ and NO_3^- would have been expelled from the membrane, and the current within the membrane conducted entirely by the K^+ and Cl^- ions migrating in opposite directions. The current was furnished by storage cells and regulated by a finely graduated ballast resistance. As a result of polarization taking place at the platinum electrodes the current always tends to fall during the first minutes of the experiment but it is easy to maintain a current of constant intensity (at least within ½ to 1 per cent of the total value) by watching a sensitive milliammeter and varying the amount of ballast resistance by hand. When the membrane itself does not cause trouble due to injury by the current, the necessary change in the applied resistance is quick only in the first few minutes, then a gradual and uniform regulation is required, and finally no essential variation of the resistance is necessary even for a long period.

In the first experiments membranes of the bag form were employed. The electrode inside the bag was a platinum spiral; for the outside electrode a large platinum net bent in such a way as to surround at least half the circumference of the bag was used. It was always kept at a reasonable distance from the membrane (2–3 cm.). The following observations were made.

1. The total quantity of current (calculated from product of milliamperes and seconds). From this the total amount of ionic transfer could be computed from the formula

1 milliampere × 1 minute = 0.000622 milli-equivalents of ions.

The accuracy of this method was checked several times by the use of Herroun's iodine coulometer as recommended by Ostwald and Luther[2] and found to be excellent. With the small amounts of current used in the experiments it proved more satisfactory and convenient than any form of coulometer.

2. The quantity of potassium transferred into the NaCl solution.

3. The quantity of chlorine transferred into the KNO_3 solution.[3]

4. In some cases the amount of acid developed in the anode chamber. In these experiments the assumption is necessary that practically all of the current is carried by the ions of the electrolyte and that the water ions do not participate to an appreciable extent even within the membrane. Under this assumption the quantity of H^+ ions developed in the anode chamber must correspond to the total quantity of current (milliamperes × seconds) and be equal to the sum of the K and Cl transferred. If it were true that a portion of the current was carried by the water ions this equality would no longer hold for the change of acidity would take place not only at the platinum electrodes but also at the membrane (Bethe-Toropoff effect).[4] This

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[2] Ostwald, W., and Luther, R., Hand- und Hilfsbuch zur Ausfuhrung physikochemischer Messungen, Leipsic, 1925, 4th edition, 569.

[3] A slight modification of the procedure for determining Cl described in the first paper of this series seemed useful. The solution, which as a rule was acid as a result of the electric transfer, was slightly alkalinized by KOH to a slight pink with phenolphthalein, evaporated almost to dryness, acidulated with just sufficient N/10 acetic acid to decolorize the phenolphthalein, then treated with potassium chromate as before described. Approaching the end-point of the titration with N/100 $AgNO_3$, after each drop of silver solution the liquid was centrifugalized. Under these conditions the first trace of a precipitate of silver chromate could be recognized as a brownish layer above the white AgCl. The increased labor involved in the centrifuging brings increased accuracy. 1 drop of excess of $AgNO_3$ N/100 results in a striking brown layer.

[4] The most important paper of Bethe and Toropoff (4) belongs to the same class of phenomena as the Hittorf effect. They are concerned only with the changes in concentrations of H^+ and OH^- ions on both sides of a membrane

effect can be of appreciable magnitude only in very dilute solutions. In our experiments the agreement obtained between total current and sum of migrated electrolyte ions was close enough to indicate that neglecting the Bethe effect introduced no error of greater magnitude than that involved in the chemical analyses.

4. THE EXPERIMENTAL RESULTS.

A. Check of the Method.

The most frequently used arrangement of apparatus in the experiments was as follows:

Large platinum net (anode)	0.2 M KNO_3	Membrane	0.2 M NaCl	Large platinum spiral (cathode)

The intensity of the current was measured with a milliammeter and regulated with a variable resistance. The first problem was to check the soundness of the method by proving that the amount of chlorine transferred in one direction and K in the other was equivalent to the total transfer as estimated from the coulombs applied. It is obvious that no K can be lost during the experiment but it is just possible that a portion of the Cl entering the KNO_3 solution might be oxidized at the electrode and removed from the solution. In view of the small number of Cl^- ions in the anode chamber relative to the NO_3^- ions, even at the end of the experiment, it does not seem likely that any appreciable amount could be set free at the electrode. Nevertheless, it seems desirable to prove by experiment that the small quantities of Cl found are in reality due to the fact that Cl plays only a minor rôle in carrying the current and not to a loss of Cl.

Table I lists the results of two experiments in which the total ionic equivalent calculated from the coulombs passed is compared with the sum of K and Cl found by analysis. The agreement is very good in view of the analytical difficulties involved and quite sufficient to prove that no appreciable amount of Cl is lost. It also shows that any current transported by water ions (Bethe-Toropoff effect) is too

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brought about by an electric current. This change of pH which we called the "Bethe-Toporoff effect" occurs in an appreciable amount only in very dilute electrolyte solutions.

small to produce a measurable error. Having shown that this agreement holds we are justified in using analytical figures for Cl in experiments in which the amount of K or analogous cation was not or could not be determined and the total ionic transfer estimated only from the coulombs passed.

TABLE I.

Potassium Titrations (1/3 of Total Quantity Taken for Analysis).

Experiment No.	N/50 Na oxalate cc. used	KMnO$_4$ solution	Corrected by factor to N/100	N/100 KMnO$_4$ used	Millimols K	Total K found	Average corrected for K in reagents	Total transfer	
								According to the analyses for K and Cl	According to the coulombs passed
135 (1)	1.40	4.12	4.34	2.94	.01070	.03210	.03136	.03744	.0373
135 (2)	.70	3.86	4.07	3.37	.01226	.03678			
136 (1)	.80	3.86	4.07	3.27	.01190	.03570	.03424	.03982	.0373
136 (2)	.60	3.88	4.08	3.48	.01265	.03795			
Check analyses to determine necessary correction for amount of potassium in NaCl used. .035 millimols K added to 50 cc. N/5 NaCl (amount used in above experiments). 1/3 of total taken for analyses.									
1	.60	4.11	4.33	3.73	.01357	.04071	Average = .03758		
2	1.10	4.11	4.33	3.23	.01175	.03525	Added K present = .03500		
3	.90	4.06	4.27	3.37	.01226	.03678	K in reagent = .00258		

Table to show accuracy of agreement between milli-equivalents calculated from the current passed and actual transfer of ions as determined by analysis. N/5 KNO$_3$ solution surrounding positive electrode. N/5 NaCl solution surrounding negative electrode. Solutions separated by C_1 collodion membrane. Current strength = 0.01 ampere for 6 minutes. Total milli-equivalents of current = .03732 (short time used to avoid error due to diffusion).

In four identical experiments Cl$^-$ transferred was .0054; .0054; .0063; and .0052 millimols, respectively. Average figure of .00558 used in calculation of total transfers.

In some of the later protocols similar checks will be found but special care was taken in the experiments of Table I. In these experiments the solutions were in contact with the membrane for less than 7 minutes (total current time 6 minutes) and the time was too short for a measurable error due to spontaneous diffusion. In other experiments where the current intensity was smaller and the current time was longer, a small correction was necessary for K spontaneously diffused, disturbing somewhat the accuracy of agreement. In none of the experiments could we show that a significant amount of Cl migrated by spontaneous diffusion. Therefore this factor can play no part in disturbing the accuracy of the transfer number for Cl when it is calculated from the Cl found by analysis and the total transfer estimated from the current applied.

B. Protocols of Transfer Experiments.

1. Bag Membrane 31.

a. Characteristics of the Membrane.—*Co P* before the transfer experiment = 52.0 millivolts. After the experiment the membrane was washed in distilled water for 3 days and then the following successive P.D. measurements made.

1. 0.01 M KCl on both sides of membrane...............2.3 millivolts.
2. 0.1 M KCl on both sides of membrane...............0.0 millivolt.
3. 0.01 M KCl on both sides of membrane.. 0.0 millivolt. (This reading made after solutions had been in contact with membrane for 30 minutes.)
4. *Co P*...47.1 millivolts.

b. The Transfer Experiment.—Outside solution 0.1 M KNO_3; inside solution 0.1 M NaCl; current strength 0.500 milliamperes; time 70 minutes.

K^+ transferred (analysis of NaCl solution)......0.0182 milli-equivalents.
Cl^- transferred (analysis of KNO_3 solution).....0.0045 milli-equivalents.
Total observed transfer ($K^+ + Cl^-$)...........0.0227 milli-equivalents.
Total expected transfer.......................0.0218 milli-equivalent.

$$t_{Cl^-} = \frac{Cl^-}{K^+ + Cl^-} = 0.20$$

c. Agreement with Transfer Number Determined by the Concentration Chain Method.—After the transfer experiment P.D. measurements were made of the membrane with the following solutions and the transfer numbers calculated according to the method previously described.

0.05 M KCl : 0.1 M KCl – P.D. = 12.8 mv. at 24°C. $t^- = 0.14$
0.1 M KCl : 0.2 M KCl – P.D. = 9.8 mv. at 24°C. $t^- = 0.22$

The transfer number observed in the transfer experiment (0.20) lies between the value of the two average transfer numbers calculated from the concentration chains; *i.e.*, $0.14 < 0.20 < 0.22$.

2. Bag Membrane 100.

a. Characteristics of the Membrane.—*Co P* before the transfer experiment = 52.5 millivolts. Following the experiment and after abolition of the polarization potential *Co P* was only 42.3 millivolts.

b. The Transfer Experiment.—Outside solution 0.01 M KNO_3; inside solution 0.01 M NaCl; current strength 0.125 milliampere; time 150 minutes.

K^+ transferred	0.0095 milli-equivalents.
Cl^- transferred	0.0009 milli-equivalents.
Total observed transfer ($K^+ + Cl^-$)	0.0104 milli-equivalents.
Total expected transfer	0.0117 milli-equivalents.

$$t_{Cl^-} = \frac{Cl^-}{K^+ + Cl^-} = 0.08$$

c. Agreement with Transfer Number Determined by the Concentration Chain Method.—Following the experiment the P.D. of the membrane was measured in the following concentration chains.

KCl 0.005 M : 0.01 M – P.D. = 16.3 mv. at 24°C. $t^- = 0.039$
KCl 0.01 M : 0.02 M – P.D. = 13.2 mv. at 24°C. $t^- = 0.126$

The value of t^- as observed in the transfer experiment lies between the values of the two average transfer numbers calculated from the concentration chains; *i.e.*, $0.039 < 0.08 < 0.126$.

3. Bag Membrane 47.

a. Characteristics of the Membrane.—*Co P* before the transfer experiment = 54.3 millivolts. *Co P* after the experiment and after abolition of polarization potential = 50.3 millivolts.

b. The Transfer Experiment.—Outside solution 0.01 M KNO_3; inside solution 0.01 M NaCl; current strength 0.125 milliampere; time 150 minutes.

K^+ transferred	0.0118 milli-equivalents.
Cl^- transferred	<0.0016 milli-equivalents.[5]
Total observed transfer ($K^+ + Cl^-$)	<0.0134 milli-equivalents.
Total expected transfer	0.0117 milli-equivalents.

$$t_{Cl^-} = \frac{Cl^-}{K^+ + Cl^-} < 0.14$$

c. Agreement with Transfer Number Determined by the Concentration Chain Method.—Following the experiment the P.D. of the membrane was measured in the following concentration chains.

KCl 0.005 M : 0.01 M – P.D. = 16.1 mv. at 24°C. $t^- = 0.046$
KCl 0.01 M : 0.02 M – P.D. = 15.1 mv. at 24°C. $t^- = 0.074$

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[5] The amount of Cl^- here represented corresponds to 4 drops of the 0.01 M $AgNO_3$ used in titrating and is too small for accuracy. The quantity recorded must be considered as a maximum value and is probably too high.

The value of t^- as observed in the transfer experiment is slightly greater than that estimated from the concentration chains but of the same order of magnitude and as such, in view of the analytical difficulties, in accordance with expectations.

4. *Flat Bell Jar Membrane C_1.*

The results of a long series of transfer experiments with this membrane have been arranged in Table II.

5. DISCUSSION.

The transfer experiments reported in this paper are confirmatory of the results obtained by the indirect method previously described (1), namely that in the membrane the transfer number of the anion is much smaller than that of the cation and that this difference is the most marked in the more dilute solutions. The finding of a membrane with such durable properties as the type with which the experiments of Table II were performed made it possibe to carry out a long series of experiments in which the effect of varying the cation and the concentrations could be observed. Here again it will be seen that the transfer number of Cl^- in solutions of equal concentration but with different cations is in the order Li > Na > K > H. Moreover, the transfer numbers regularly become greater as the concentration of the electrolytes is increased. This membrane was also used in a series of 2:1 concentration chain experiments with KCl (1) and it is possible to compare the transfer numbers in the different ranges of concentration as estimated by this method with those actually found in the transfer experiments. The result is shown in Fig. 1. On the whole the agreement is good. Whether the minor discrepancies are the result of some systematic and unrecognized error or due to an additional unknown factor not taken into account in the theory is not clear. In this connection it is desirable to call attention to an observation which was made during the series of experiments and for which no satisfactory explanation is as yet apparent. When the membrane was used in a short space of time for several successive transfer experiments, almost invariably it was found that the transfer number for Cl^- calculated from the results of the first experiment was somewhat higher than the transfer numbers computed from the succeeding experiments. Had the reverse

TABLE II.

Experiment No.	Solution in anode compartment	Solution in cathode compartment	Current strength	Time	Calculated total transfer	Millimols K found	Millimols Cl found	Total transfer found	Transfer No. Cl^-
			milliamperes	*min.*	*milli-equivalents*				$\frac{Cl^-}{\text{calculated total transfer}}$
A	M/50 KNO_3	M/50 NaCl	2	15	.01866	.01812	.0023	.02042	0.123
2	M/50 KNO_3	M/50 NaCl	2	30	.03732		.0045		0.121
3	M/50 KNO_3	M/50 NaCl	2	30	.03732		.0030		0.080
8	M/50 KNO_3	M/50 KCl	2	30	.03732		.0039		0.104
143	M/50 KNO_3	M/50 KCl	6	10	.03732		.0045		0.121
144	M/50 KNO_3	M/50 KCl	6	10	.03732		.0020		0.054
145	M/50 KNO_3	M/50 KCl	6	20	.07464		.0045		0.060
30	M/10 KNO_3	M/10 NaCl	2	35	.04354	.0402	.0043	.04452	0.095
31	M/10 KNO_3	M/10 NaCl	2	30	.03732	.0405	.0035	.04401	0.094
37	M/5 KNO_3	M/5 NaCl	2	30	.03732	.0393	.0043	.04362	0.116
39	M/5 KNO_3	M/5 KCl	2	30	.03732		.0059		0.157
40	M/5 KNO_3	M/5 KCl	2	32	.03981		.0048		0.122
42	M/5 KNO_3	M/5 NaCl	4	15	.03732	.0360	.0053	.04135	0.143
43	M/5 KNO_3	M/5 NaCl	4	15	.03732	.0374	.0047	.04215	0.127
133	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0414	.0054	.04680	0.145
134	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0388	.0054	.04420	0.145
135	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0319	.0063	.03816	0.169
136	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0342	.0052	.03944	0.139
146	M/5 KNO_3	M/5 KCl	6	20	.07464		.0175		0.234
147	M/5 KNO_3	M/5 KCl	6	20	.07464		.0100		0.134
148	M/5 KNO_3	M/5 KCl	6	20	.07464		.0085		0.114
34	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0076		0.205

33	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0068		0.183
131	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0077		0.206
132	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0099		0.265
47	M/1 KNO_3	M/1 NaCl	4	15	.03732		.0108		0.290
139	M/1 KNO_3	M/1 KCl	6	10	.03732		.0150		0.402
140	M/1 KNO_3	M/1 KCl	6	11	.04105		.0164		0.400
141	M/1 KNO_3	M/1 KCl	6	10	.03732		.0146		0.391
1	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0082		0.220
6	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0065		0.174
7	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0057		0.152
13	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0100		0.268
14	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0077		0.206
15	M/50 $LiNO_3$	M/50 NaCl	2	30	.03732		.0131		0.349
16	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0099		0.266
17	M/50 $LiNO_3$	M/50 LiCl	2	31	.03856		.0113		0.294
18	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0097		0.261
19	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0056		0.150
23	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0083		0.221
24	M/50 $LiNO_3$	M/50 LiCl	2	60	.07464		.0196		0.263
27	M/10 $LiNO_3$	M/10 LiCl	2	31	.03856		.0124		0.322
28	M/10 $LiNO_3$	M/10 LiCl	2	30	.03732		.0112		0.299
4	M/50 HNO_3	M/50 HCl	2	30	.03732		.0013		0.035
5	M/50 HNO_3	M/50 HCl	2	35	.04354		.0012		0.028
9	M/50 HNO_3	M/50 KCl	2	30	.03732		.0017		0.046

Tabulation of results in 46 electric transfer experiments performed with the same dried collodion membrane (C_1-flat type). Experiments were done during a period of 4 months, the constancy of the properties of the membrane being shown by frequent *Co P* determinations. *Co P* at beginning of experiments (9–28–26) 48.7 millivolts; (11–8–26) 48.5 millivolts; (12–11–26) 49.0 millivolts; at end of experiments (1–21–27) 49.0 millivolts.

been true and the later experiments given higher transfer numbers we should have supposed that the electroendosmosis had temporarily stretched the membrane pores, lowered its *Co P*, and increased its permeability for the anion. However, the observed findings were the opposite of that which might have been expected. It scarcely seems wise to attempt an explanation until more experimental data has been accumulated. However, the variations in the transfer numbers referred to here were of a minor degree and do not interfere with their interpretation in the present communication. The transfer numbers determined in these experiments correspond fairly closely to those calculated indirectly from the concentration chains and furnish an additional reason for maintaining the theory set forth in connection with the latter method. We wish, however, to emphasize that the agreement between the two methods is not complete and that we believe that the range of variation is greater than the limits of error. Obviously the theory in its present state of development is insufficient to explain all data in a really quantitative way.

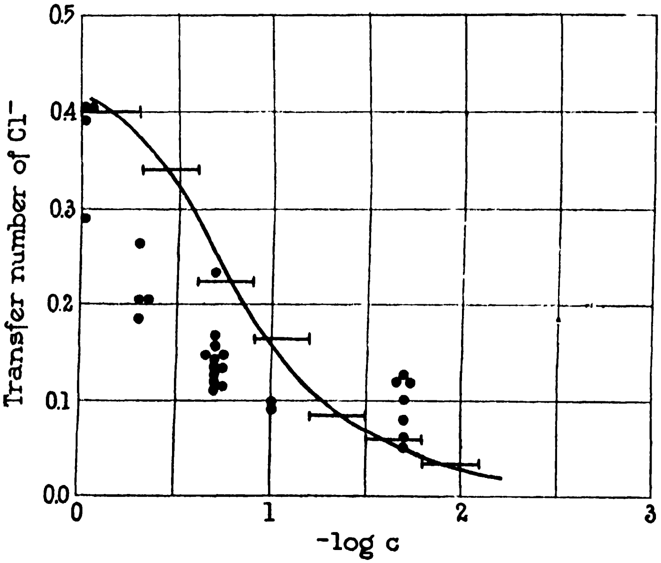

FIG. 1. Shows accuracy of agreement between chlorine transfer numbers with KCl as determined by the concentration chain method (continuous curve) and by electric transfer experiments (separate dots).

SUMMARY.

The transfer numbers of the ions of electrolytes in the dried collodion membrane, as determined in a previous paper indirectly from the E.M.F. of concentration chains, can also be determined directly by electrical transfer experiments. It is shown that the difficulties involved in such experiments can be overcome. The transfer numbers obtained by the two methods are in satisfactory agreement. The experimental results obtained in the transfer experiments furnish an additional argument in favor of maintaining the theory that the electromotive effects observed in varying concentrations of different electrolytes with the dried collodion membrane may be referred to differences in the mobilities of the anions and cations within the membrane. As was shown by the method of the previous paper, the transfer number depends largely on concentration. There are some minor discrepancies between the values of the transfer numbers obtained by the two methods which, as yet, cannot be completely explained.

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THE ACTIVATION OF STARFISH EGGS BY ACIDS.

II. The Action of Substituted Benzoic Acids and of Benzoic and Salicylic Acids as Influenced by Their Salts.

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INTRODUCTION.

In a recent paper[1] I described experiments indicating that the molecular rate of action of penetrating and parthenogenetically effective acids (fatty acids, carbonic acid, benzoic acid) is determined primarily by their strength as acids. In a pure solution of any single acid (within the range of concentrations effective in activation) the activation process within the egg proceeds at a rate which is closely proportional to the concentration of acid. If, comparing different acids, we consider solutions having as nearly as possible the same physiological effect, *e.g.*, causing complete activation in 10 minutes at 20°, we find a significant relationship: namely, the square root of the product of the dissociation constant into the concentration of acid appears nearly constant. Since the C_H shows a similar proportionality in solutions of weak acids in the absence of their salts ($C_H = \sqrt{K \times C_{\text{acid}}}$), this relationship might seem to indicate that the C_H of the external solution is the essential factor determining the rate of action. This, however, is shown not to be the case by the effects following the addition of the sodium salt of the acid to the solution. For example, the addition of .002 M Na acetate to the .002 M solution of acetic acid lowers the ionization of the acid—from *ca.* 10 per cent to *ca.* 1.5 per cent—thus bringing the C_H nearer to neutrality and correspondingly increasing the concentration of undissociated molecules. The fact that the rate of activation is *increased* by this addition, and to a degree (*ca.* 10 per cent) corresponding closely to the estimated increase in the undissociated molecules of acid, indicates that the essential factor in the activating effect is the concentration of the undissociated free acid in the external solution, and not the concentration of the ions of the acid. Apparently the undissociated acid molecules penetrate the egg rapidly and dissociate in its interior, serving there as a source of H ions; these then in some way induce activation, at a rate proportional to their concentration. The observed relationship between the strength of the acid and its activating effectiveness is thus explained. The rate of the activation reaction, within the appropriate range of C_H, would thus seem to be directly proportional to the C_H at the site of the activation reaction inside the egg, apparently in the cortical region of the latter.

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[1] Lillie, R. S., *J. Gen. Physiol.*, 1925–27, viii, 339.

Since this relation is an interesting one, I have continued these experiments during the past summer, using several additional acids. The number of acids available for this purpose is not large. Many apparently effective acids which cause typical membrane formation and early cleavage have a secondary toxic action and the eggs die before reaching the blastula stage. This is the case, for example, with caprylic acid and the three chloroacetic acids; and during the past summer the same was found true for phthalic acid. The membrane-forming action, taken alone, has a certain value as an index of the relative physiological effectiveness of acids; thus the relative rates of action of mono-, di-, and trichloroacetic acids as thus estimated are in the order of their dissociation constants;[1] but this test is a less satisfactory one than the production of blastulæ in a large proportion of eggs, and the latter index has been mainly relied upon in these experiments.

It seemed probable that the question of the relation between strength and activating efficiency could best be tested by using a series of acids varying in strength but similar in their constitution and presumably also in their ability to penetrate the egg. Since benzoic acid had proved highly effective as an activating agent in low concentrations (.00025 M to .001 M), experiments with a series of substituted benzoic acids were tried, including phthalic, salicylic, *o*-, *m*-, and *p*-chlorobenzoic, and *o*-, *m*-, and *p*-nitrobenzoic. These acids are relatively non-toxic in the low concentrations used, and have a wide range of dissociation constants. The following are the values given in the Landolt-Börnstein Tables:[2]

TABLE I.

Acid	Dissociation constant
Benzoic	6×10^{-5}
Salicylic	1.06×10^{-3}
Phthalic (first constant)	1.26×10^{-3}
o-Chlorobenzoic	1.32×10^{-3}
m-Chlorobenzoic	1.55×10^{-4}
p-Chlorobenzoic	9.3×10^{-5}
o-Nitrobenzoic	6.3×10^{-3}
m-Nitrobenzoic	3.5×10^{-4}
p-Nitrobenzoic	4.0×10^{-4}

The method used was the same as in the previous series. The acids were dissolved in isotonic NaCl-$CaCl_2$ solution (100 volumes 0.52 M NaCl + 5 volumes 0.5 M $CaCl_2$). 100 cc. of a given solution of acid (at 20°) was added to 1 or 2 cc. of a dense suspension of the eggs in sea water, and at regular intervals (of 1 or 2 minutes) eggs were transferred in serial order to bowls containing sea water. Later the eggs were examined and the proportions developing to a swimming stage (blastula or gastrula) were determined.

Results with Different Acids.

The following summaries give the essential results obtained with pure solutions of the various acids.

Benzoic Acid.—The results previously obtained with this acid were confirmed. Using the concentrations, .0004 M, .0005 M, and .0006 M, the characteristic optimum exposures were, respectively, 12 to 16 minutes, 10 to 12 minutes, and *ca.*8 minutes. A large majority of eggs thus exposed (varying from 70 to 90 per cent in different experiments) formed blastulæ.

Phthalic Acid.—The concentrations used were .0002 M, .0003 M, .0004 M, and .0006 M, with exposures ranging from 2 to 20 minutes. This acid proved ineffective as an activating agent, apparently because of some secondary toxic action. It forms typical membranes, but the eggs break down before reaching swimming stages. The minimum exposures required to form membranes in 90 per cent (or more) of the eggs were (for the above four concentrations), respectively, 10, 6, 4, and 2+ minutes. The rate of membrane-forming action is thus closely proportional to concentration; it is, however, slower than that of benzoic acid, which in concentrations so low as .00025 M forms membranes in nearly all eggs with exposures of 2 to 3 minutes. This relatively gradual action of phthalic acid, as compared with the weaker benzoic acid, may be an indication of slower penetration, and has its parallel in the slowness of action of *o*-nitrobenzoic acid. The specific toxicity of the acid is an independent property, apparently analogous to that of oxalic acid.

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[2] Landolt, H., and Börnstein, R., Tabellen, Berlin, 5th edition, 1923, 1138 *et seq.*

Salicylic Acid.—In appropriate concentrations (.0002 M to .0006 M) this acid causes typical activation in a large proportion of eggs. Its molecular rate of action is between two and three times that of benzoic acid; its toxicity also is distinctly greater. An exposure of 2 minutes to the .0002 M solution is sufficient for membrane formation; exposures of 14 to 16 minutes (the optimum for .0002 M) cause most eggs to form blastulæ. Stronger solutions act more rapidly, but less favorably. Within the above range the rate of action is approximately proportional to concentration. The following is a summary of observations made during June, 1926, with good controls (Table II).

TABLE II.

Salicyclic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of eggs forming blastulæ
M	
.0002	(*7 series*) 16 min. (20–25 per cent); 14–16 min. (*ca.* 50 per cent); 13–15 min. (35–45 per cent); 16–18 min. (65–70 per cent); 12–14 min. (50–60 per cent); *ca.* 16 min. (70–80 per cent); 14 min. (35–45 per cent)
.0003	(*2 series*) 8–10 min. (30–40 per cent); 7–6 min. (20–30 per cent)
.0004	(*3 series*) 8–10 min. (5–10 per cent); 8–10 min. (30–40 per cent); 5–6 min. (25–30 per cent)
.0006	(*1 series*) 2–4 min. (few)

Chloro- and Nitrobenzoic Acids.—With the exception of *o*-nitrobenzoic acid, all the acids of this group activate more rapidly than benzoic acid. In the case of the meta- and para-acids the greater rate of action corresponds closely with that calculated from the dissociation constants. It is remarkable, however, that both ortho-acids, particularly *o*-nitrobenzoic acid, are much less effective than their very considerable strength as acids would lead us to expect. The following summaries give the chief observations with the single acids.

o-Chlorobenzoic Acid.—This acid is somewhat more rapid in its action than benzoic acid; its specific toxicity is also greater, and fewer eggs form blastulæ at the optimum exposures. The following series (Table III) is typical (June 18, 1926).

m- and p-Chlorobenzoic Acids.—These acids are closely similar in their rates of action. On account of their slight solubility the concentrations used were limited to .00025 M and .00035 M for the *m*-acid, and .00025 M for the *p*-acid.[3] The following results are typical (Table IV).

All three chlorobenzoic acids act more rapidly than benzoic acid, but the ortho-acid is decidedly slower in its action than the other two, which are closely similar. This difference is illustrated in the series summarized in Table V, in which equimolecular solutions (.00025 M) of the four acids were compared in their action on the same lot of eggs.

TABLE III.

o-Chlorobenzoic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of blastulæ
M	
.0002	*Ca.* 34 min. (30–40 per cent)
.0003	22–26 min. (35–45 per cent)
.0004	12–14 min. (20–25 per cent)
.0005	*Ca.* 8 min. (20–25 per cent)

TABLE IV.

m- and p-Chlorobenzoic Acids. Temperature 20°.

Solution	Optimum exposures and percentages of blastulæ
M	
(*m*-Chlorobenzoic) .00025	(*1 series*) 7–8 min. (35–45 per cent)
.00035	(*1 series*) 3–4 min. (30–40 per cent)
(*p*-Chlorobenzoic) .00025	(*3 series*) 7–9 min. (25–35 per cent); 7–8 min. (30–40 per cent) 9–11 min. (65–75 per cent)

Ortho-chlorobenzoic acid, although the strongest of the three chloro-acids, acts more slowly than the meta- and para-acids and apparently is somewhat more toxic; of the two latter the meta-acid is somewhat the more effective. This unexpected behavior of the ortho-acid seems to indicate some difficulty in penetration, as in the analogous case of *o*-nitrobenzoic acid.

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[3] The concentrations of the saturated solutions in distilled water were determined by titration with N/50 NaOH, using brom thymol blue as indicator. The solutions in isotonic NaCl-$CaCl_2$ were made by diluting the distilled water solution with a concentrated NaCl-$CaCl_2$ solution of a strength such that when the dilution was made the resulting solution was isotonic.

o-Nitrobenzoic Acid.—This acid, the strongest of the group, is somewhat surprisingly ineffective. Exposures up to 14 minutes, with the concentrations .0002 M, .00025 M, .0003 M, .0004 M, and .0005 M, gave only slight activation in any case. The longest exposure (14 minutes) to the .0005 M solution resulted in less than 1 per cent of blastulæ. The membrane-forming action is also gradual, indicating slow penetration; the exposures required to form membranes in 50 per cent of eggs, with the concentrations .0002 M, .0003 M, and .0004 M, were, respectively, 7 to 9 minutes, 6 to 7 minutes, and 4 to 5 minutes. Membranes are formed by the .0002 M solutions of the other substituted acids and benzoic acid in 2 to 3 minutes.

TABLE V.

Benzoic and Chlorobenzoic Acids. Temperature 20°.

Acid	Optimum exposures and percentages of blastulæ
.00025 M	
Benzoic	Longest exposure 24 min. (20–30 per cent); optimum not reached
o-Chlorobenzoic	Optimum not reached; *ca.* 5 per cent blastulæ at 12 min.
m-Chlorobenzoic	7–8 min. (35–45 per cent)
p-Chlorobenzoic	9–11 min. (65–75 per cent)

The exposures were 1 to 24 minutes for benzoic acid and 1 to 12 minutes for the other three acids; eggs were transferred to sea water at intervals of 1 minute.

TABLE VI.

m-Nitrobenzoic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of blastulæ
M	
.0001	(*1 series*) few membranes formed up to 30 minutes exposure
.0002 .00025	(*3 series*) 18–24 min. (40–50 per cent); 24–28 min. (*ca.* 50 per cent); 24–28 min. (*ca.* 50–60 per cent)
.0003	(*3 series*) 12–16 min. (30–40 per cent); 16–18 min. (40–50 per cent); *ca.* 6 min. (30–40 per cent)
.0004	(*5 series*) 10–12 min. (*ca.* 50 per cent); 8–12 min. (30–40 per cent); 8–10 min. (30–35 per cent); 5–7 min. (10–15 per cent); *ca.* 4 min. (25–35 per cent)
.0005	(*3 series*) *ca.* 6 min. (20–25 per cent); 5–6 min. (20–25 per cent); 4–5 min. (*ca.* 10 per cent)

m-Nitrobenzoic and p-Nitrobenzoic Acids.—A summary of the observations with these acids is given in Tables VI and VII. The two are closely similar in their action, with the *p*-acid slightly more rapid and slightly more toxic than the *m*-acid.

The differences between benzoic acid and the nitrobenzoic acids are brought out clearly in the series summarized in Table VIII, in which the four acids in equal concentrations (.00025 M) were used with the same lot of eggs.

It is clear that nitro-substitution in the meta- and para-positions greatly increases the effectiveness of benzoic acid. The same is true of chlor-substitution. The relative ineffectiveness of *o*-nitro acid is difficult to understand, but is probably connected with its relatively great strength as an acid ($K = 6.3 \times 10^{-3}$). It is stronger than salicylic acid ($K = 1.06 \times 10^{-3}$) and formic acid ($K = 0.22 \times 10^{-3}$) and decidedly stronger than lactic acid ($K = 0.14 \times 10^{-3}$) which also is

TABLE VII.

p-Nitrobenzoic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of blastulæ
M	
.0002	(*1 series*) 16–18 min. (20–25 per cent)
.00025	(*1 series*) 9–10 min. (30–35 per cent)
.0003	(*3 series*) 8–12 min. (10–20 per cent); *ca.* 6 min. (15–25 per cent); 4–5 min. (25–30 per cent)
.0004	(*3 series*) 6–8 min. (10–20 per cent); 3–4 min. (15–20 per cent); 3–4 min. (20–25 per cent)
.0005	(*3 series*) 3–4 min. (15–20 per cent); 4–6 min. (5–10 per cent); *ca.* *3* min. (15–20 per cent)

TABLE VIII.

Benzoic Acid and Nitrobenzoic Acids. Temperature 20° (July 26, 1926).

Acid	Optimum exposures and percentages of blastulæ
.00025 M	
Benzoic	30 min. + (50 per cent at 30 min., the longest exposure)
o-Nitrobenzoic	Action slow; 7 min. required to form membranes in 50 per cent of eggs; almost no blastulæ formed up to 12 min.
m-Nitrobenzoic	11–12 min. (35–50 per cent)
p-Nitrobenzoic	9–10 min. (30–35 per cent)

With benzoic acid eggs were transferred to sea water at 2 minute intervals, from 2 to 30 minutes; with the other acids at 1 minute intervals, from 1 to 12 minutes.

ineffective as compared with the fatty acids. Of the first five fatty acids formic acid is the least effective in relation to the C_H of its solutions,[4] a peculiarity probably related to its greater strength. The comparative ineffectiveness of the mineral acids is well known; in this case the difficulty is one of penetration. The indications from membrane formation are that ortho-nitrobenzoic and lactic acids also penetrate the egg slowly. The factors determining the rate of penetration are insufficiently known at present. Surface activity is undoubtedly important, but the relation to ionization as such is uncertain, as indicated (*e.g.*) by the differences between salicylic acid and the equally strong ortho-benzoic acids.

Relation between Strength and Activating Effectiveness of Penetrating Acids.

If we again[4] compare the concentrations of pure acid solutions having equal rates of action, taking as the standard rate that corresponding to an optimum exposure in 10 minutes at 20°, the values (approximate) given in Table IX are obtained. This table includes the results of the earlier experiments with fatty acids and carbonic acid[1] as well as those of the present series. The second column gives the concentrations of the physiologically equivalent solutions, and the last column the calculated H ion concentrations of these solutions. While some degree of irregularity is shown, the C_H values are evidently closely similar; with the exception of formic acid and the two ortho-benzoic acids, all lie between 1.1 and 2.1×10^{-4} N and the majority between 1.6 and 2.1×10^{-4} N (pH = 3.7–3.8). The relation between the strength of the activating acids and their rate of action is thus a direct one, in conformity with the general idea that the rate of the activation reaction is determined by the intracellular C_H. We may infer that activation begins when a certain critical C_H is attained at the localized site of the reaction (apparently in the egg cortex) and proceeds at a rate which is closely proportional to this C_H. The reaction reaches its completion in a time determined by C_H and temperature, and in some way transforms the egg from the resting to the automatically developing state.

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[4] Lillie,[1] Table XII, p. 364.

These estimates of C_H are subject to correction, since they do not take account of the increased dissociation which the weak acid almost certainly undergoes in the presence of the neutral salt; the possibility should also be considered that there are other factors within the egg influencing dissociation, such as the electrically polarized state of the structural surfaces at or near which the activation reaction occurs. The precise degree of the neutral salt influence is as yet unknown; but the physiological effects indicate that in the case of acetic, benzoic, and salicylic acids dissolved in 0.5 M NaCl it is equivalent to a twofold or threefold increase in the dissociation constant.[5] This is clearly seen in the manner in which the action of the acid varies in the presence of varying concentrations of its salt (*cf.* next section).

TABLE IX.

Approximate Concentrations of Acids Causing Complete Activation in 10 Minutes at 20°.

Acid	Concentrations	Dissociation constants	Calculated C_H of solutions
	M		($C_H \times 10^4$)
Formic	.0008	2.2×10^{-4}	4.2
Acetic	.0025	1.8×10^{-5}	2.1
Propionic	.0024	1.4×10^{-5}	1.8
Butyric	.0022	1.4×10^{-5}	1.75
Valeric	.0018	1.4×10^{-5}	1.6
Caproic	.0014	1.45×10^{-5}	1.4
Carbonic	.035	3.2×10^{-7}	1.1
Benzoic	.0005	6×10^{-5}	1.7
Salicylic	.00025	1.06×10^{-3}	2.1
o-Chlorobenzoic	.00045	1.32×10^{-3}	3.5
m-Chlorobenzoic	.0002	1.55×10^{-4}	1.15
p-Chlorobenzoic	.00025	0.93×10^{-4}	1.12
o-Nitrobenzoic	(?)	6.3×10^{-3}	(?)
m-Nitrobenzoic	.00035	3.5×10^{-4}	2.1
p-Nitrobenzoic	.00025	4.0×10^{-4}	1.7

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[5] As just indicated, it is possible that the salt influence in the interior of the egg may be supplemented by other factors peculiar to the egg system. For example, the orientation of the adsorbed molecules of acid at the structural interfaces, with the carboxyl groups directed toward the water phase, as Harkins' theory requires, may increase dissociation, very much as various terminal substitutions are known to do. Combined with this influence would be that of the electrical polarization field at the phase boundary.

Action of Acids in Presence of Their Salts.

It was shown in the previous paper[1] that when Na acetate (.002 to .016 M) is added to activating solutions of acetic acid (.002 to .004 M) the rate of activation is increased to a moderate degree (10 to 20 per cent), in correspondence with the estimated proportional increase in undissociated acetic acid. Experiments with strong acid (HCl)and Na acetate (added separately to the isotonic NaCl-$CaCl_2$ solution) showed that the simple addition of H ions or acetate ions is ineffective, relatively or absolutely. Apparently activation is a consequence of the penetration of the undissociated molecules of acid followed by dissociation within the egg. The fact that the addition of acetate, which decreases the ionization of the acid in the external solution, does not retard but on the contrary accelerates the action of the acid within the egg is in itself an indication of the non-penetration (or very slow penetration) of the acetate ions.[6]

Experiments performed last summer with solutions of benzoic acid *plus* Na benzoate and of salicylic acid *plus* Na salicylate bear out this interpretation. On account of the greater strength of these acids, the addition of the Na salt to their solutions causes a greater proportional increase of undissociated molecules than in the case of acetic acid. This increase is greater with salicylic acid than with benzoic acid; in correspondence with this difference, the accelerating effect of the addition of the Na salt was found much greater with the former acid (*cf.* Fig. 1). The observed acceleration shows in both cases a satisfactory correspondence with that anticipated on the assumption that only the undissociated molecules in the solution are parthenogenetically effective. The acceleration, however, was in both cases greater than that calculated on the basis of the accepted dissociation constants, a result probably attributable mainly to the increased dissociation of the acid in the presence of the neutral salt (= 0.52 M NaCl).

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[6] Some years ago, in a study of the toxic action of acids on infusoria (*Paramecium* and *Euplotes*), Miss Collett found that after the addition of non-toxic quantities of the salts of various weak acids (fatty acid, benzoic and salicylic acids) to toxic solutions of the same acids the resulting mixture was more toxic than could be explained by the acidity alone; and she interpreted this result as indicating toxicity of the undissociated acid molecules. In the case of salicylic acid and (in part) benzoic acid toxicity was definitely increased by adding the salt. These results also are in harmony with the view that only the undissociated molecules penetrate the cell freely (Collett, M. E., *J. Exp. Zool.*, 1921, xxxiv, 67).

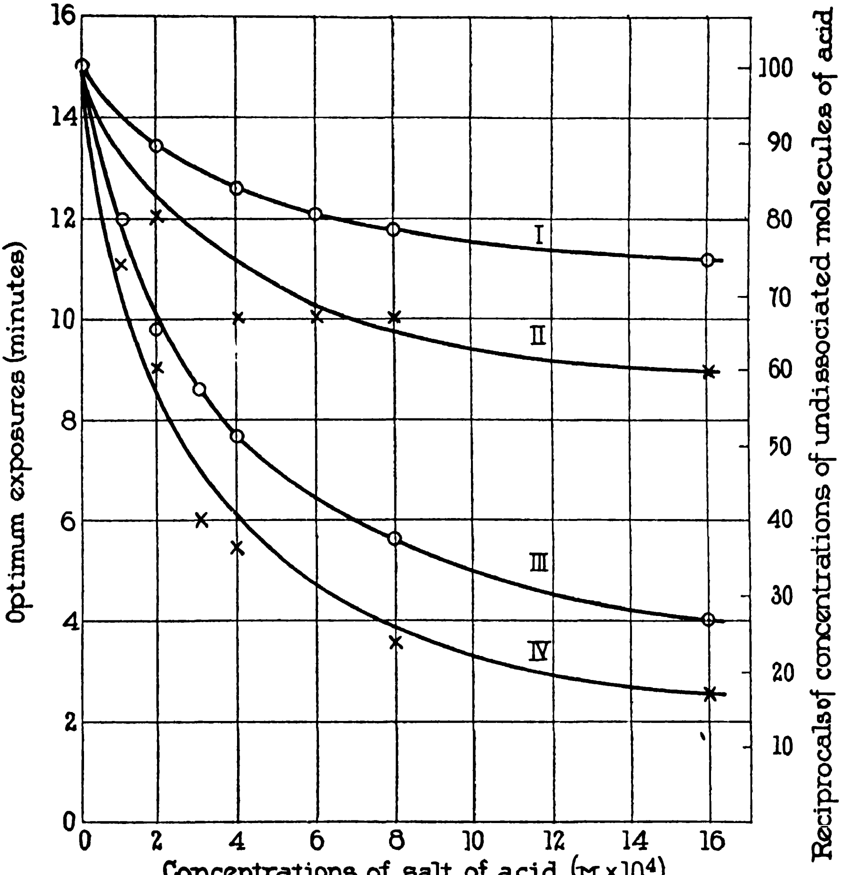

FIG. 1. Calculated and observed optimum exposures to .0004 M benzoic acid (Curves I and II) and .0002 M salicylic acid (Curves III and IV) in the presence of the Na salts of the acids. Ordinates, durations of exposures at 20°; abscissæ, concentrations of salt.

In estimating the dissociation of relatively strong acids like salicylic acid, and even of benzoic acid at the low concentrations employed in these experiments, it is necessary to take into account the anions derived from the acid, as well as those from the added Na salt, in calculating the conditions at equilibrium.[7] The C_H is given by the ratio of the concentration of undissociated molecules of acid to that of the anions (from both sources), multiplied by the dissociation constant. Since the anions and H ions derived from the acid are equal in quantity, the equation takes the form:

$$C_H = \frac{C_{\text{acid}} - C_H}{\gamma C_{\text{Na salt}} + C_H} \times K \tag{1}$$

where C is the concentration of the component indicated by the subscript, γ the degree of dissociation (activity) of the salt, and K the dissociation constant of the acid. The solution of this quadratic equation for C_H is:

$$C_H = \sqrt{\left(\frac{\gamma C_{\text{Na salt}} + K}{2}\right)^2 + KC_{\text{acid}}} - \frac{\gamma C_{\text{Na salt}} + K}{2} \tag{2}$$

The relative concentrations of dissociated and undissociated molecules in the several solutions employed, as calculated from this equation, taking γ as 0.64 and the dissociation constants of benzoic and salicylic acids as 6×10^{-5} and 1.06×10^{-3}, respectively, are given in Tables XII and XV.

Solutions of Benzoic Acid Plus Na Benzoate.—As in the series with acetic acid and Na acetate, the salt was added in a series of concentrations of the same order as that of the acid. The addition of the Na benzoate alone to the isotonic $NaCl$-$CaCl_2$ solution has no activating effect[8]; its effect when added to the solution of benzoic acid is to be attributed solely to its influence on the state of the latter.

Tables X and XI give a summary of two typical series. The benzoic acid was used in a concentration of .0004 M, giving complete activation in 14 to 16 minutes in the pure solution. In the other solutions the concentrations of benzoate ranged from .0002 M to .0016 M. It will be observed that the addition of the salt increases the rate of activation very considerably (from 25 to 70 per cent) and that the increase is relatively greater than in the similar experiments with acetic acid; also that (as in the case of acetic acid) the degree of acceleration tends somewhat rapidly toward a limit as the salt content is increased. That the accelerating influence of the salt should be greater with benzoic than with acetic acid is to be expected from the greater dissociation of this acid and the lower concentration of its effective solutions. The calculated degree of dissociation in the .0004 M solution, in the absence of benzoate, is 30 per cent (assuming $K = 6 \times 10^{-5}$). If the dissociation were completely suppressed by the addition of sufficient Na benzoate the concentration of undissociated molecules would become 100/70 of the original, an increase of *ca.* 43 per cent. This represents the limit to which increase of undissociated acid tends as the salt is added and explains why the rate of activation similarly tends toward a limit. Table XII gives the calculated dissociations of the added acid in the several solutions used (fourth column), together with the relative concentrations of undissociated acid, expressed as percentages of the free acid added (fifth column), and also (sixth column) in comparison with the undissociated

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[7] *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, pt. 1, 38. Furman, N. H., in Taylor, H. S., Treatise on physical chemistry, New York, 1924, ii, 833.

[8] The same is true of Na salicylate.

TABLE X.

Benzoic Acid Plus Na Benzoate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ										
	4	6	8	10	12	14	16	18	20	22	24
A. .0004 M benzoic acid (alone)	0	0	1–2	*Ca.* 5	20–30	*Ca.*50	70–80	70–80	50–60	15–20	*Ca.*10
B. .0004 M acid *plus* .0002 M Na benzoate	<1	20–30	40–45	65–70	60–70	30–40	25–30	*Ca.*10	*Ca.* 5	<1	0
C. .0004 M acid *plus* .0004 M Na benzoate	<1	10–15	50–60	50–60	*Ca.*50	25–35	15–20	<1	<1	0	0
D. .0004 M acid *plus* .0006 M Na benzoate	*Ca.*1	30–40	50–55	50–60	45–50	30–35	*Ca.* 1	0	0	0	0

acid in the benzoate-free solution. The reciprocals ($\times 10^4$) of the latter values are also given (seventh column).

If we assume that the rates of activation (the reciprocals of the optimum exposures) in the several solutions are directly proportional to the concentrations of undissociated acid, the observed and the expected rates of activation may be compared. This is done in the two upper curves of Fig. 1. Curve II shows the variation in the durations of the optimum exposures with varying concentrations of benzoate; the points are the approximate optimum exposures in the different solutions (*cf.* Tables X, XI, and Column 2 of Table XII). Curve I represents the reciprocals of the calculated concentrations of undissociated molecules; the concentration in the benzoate-free solution is given the value 100 (scale on right of figure) and is made to correspond with the observed optimum exposure (15 minutes) in the benzoate-free solution. If the dissociation were correctly given by the calculation, the two curves should coincide. It is evident, however, that the actual acceleration is greater in every case than that calculated. For example, the addition of .0016 M Na benzoate shortens the

TABLE XI.

Benzoic Acid Plus Na Benzoate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ										
	4	6	8	10	12	14	16	18	20	22	24
A. .0004 M benzoic acid (alone)	0	0	1–2	20–30	40–50	80–90	65–75	60–70	60–70	*Ca.*50	25–35
B. .0004 M acid *plus* .0004 M Na benzoate	<1	15–20	70–80	70–80	65–75	55–60	*Ca.*50	20–30	*Ca.* 5	0	0
C. .0004 M acid *plus* .0008 M Na benzoate	1–2	30–40	*Ca.*50	65–75	65–75	*Ca.*50	30–40	10–15	*Ca.* 5	0	0
D. .0004 M acid *plus* .0016 M Na benzoate	<1	30–40	55–60	60–70	*Ca.*50	20–30	5–10	0	0	0	0

optimum exposure from 15 minutes to *ca.* 9 minutes, an increase in the rate of activation of *ca.* 70 per cent, while the calculated increase in undissociated molecules is only 35 per cent (*cf.* Column 6, Table XII). The fact that the rate of activation is greater than that estimated is, however, readily understood if we assume that the dissociation of the acid is increased in the presence of the relatively high concentration (*ca.* 0.5 M) of neutral salt.[9] If the constant of benzoic acid is regarded as 2×10^{-4}, instead of 6×10^{-5}, the observed and the calculated curves agree closely. A similar disparity between the observed and the calculated rates of activation is seen in the experiments with salicylic acid (Curves III and IV, Fig. 1) and acetic acid.[5]

TABLE XII.

Activation by .0004 M Benzoic Acid in Presence of Na Benzoate.

Concentration of Na benzoate	Durations of optimum exposures (*ca.*) (T)	Relative rates of activation $\left(\frac{15}{T} \times 100\right)$	Acid dissociated γ	Acid undissociated $(100\text{-}\gamma)$	Relative concentrations undissociated acid $\frac{(100\text{-}\gamma)100}{70}$	Reciprocals of concentrations undissociated acid $\frac{70}{(100\text{-}\gamma)100} \times 10^4$
M	*min.*		*per cent*	*per cent*		
0	14–16 (av. = 15)	100	30	70	100	100
.0002	12	125	21.75	78.25	112	89+
.0004	10	150	15.75	84.25	120	83+
.0006	<10	150+	12.25	87.75	125	80
.0008	<10	150+	9.75	90.25	128	78
.0016	8–10 (av.9)	170	5.5	94.5	135	74

Solutions of Salicylic Acid Plus Na Salicylate.—Four series of experiments with .0002 M salicylic acid and Na salicylate ranging from .0001 M to .0016 M were performed during June, 1926, at a time when the eggs were in good and uniform condition. The results of these experiments agree closely; they all show a rapid progressive increase in the rate of activation with increasing addition of Na salicylate, the relative acceleration being decidedly greater than in the corresponding experiments with benzoic acid (Fig. 1) and tending more slowly toward a limit. Tables XIII and XIV give records of two of these series. In the absence of salicylate .0002 M salicylic acid shows an optimum effect at exposures of 14 to 16 minutes. The addition of .0002 M to .0003 M salt approximately doubles the rate of activation; that of .0008 M to .0016 M increases it from four to six times. This relatively great acceleration, as contrasted with the moderate acceleration observed with acetic and benzoic acids, is in accordance with the much higher dissociation constant ($K = 1.06 \times 10^{-3}$) of salicylic acid. A reference to the calculated percentages of dissociation given in Table XV shows that the possible increase in undissociated molecules in the .0002 M solution is from 14 per cent (in the salt-free solution) to 100 per cent (with complete suppression of dissociation),—*i.e.*, about seven times. The calculated increase resulting from the addition of .0016 M salicylate is 52/14,—nearly four times (Column 5, Table XV). The actual increase in rate of activation is, however, greater than this,—from five to six times, indicating again an increased dissociation of the acid in the neutral salt solution. A similar disparity between the observed and the calculated rates of activation is seen in every

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[9] *Cf. e.g.*, Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipzig, 6th edition, 1926, 55.

TABLE XIII.

Salicylic Acid Plus Na Salicylate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ											
	2	4	6	8	10	12	14	16	18	20	22	24
A. .0002 M salicylic acid (alone)	0	0	0	<1	*Ca.*10	*Ca.*20	35–45	60–70	50–60	40–50	20–25	10–15
B. .0002 M acid *plus* .0001 M Na salicylate	0	0	<1	15–20	35–40	45–50	*Ca.*10	5–10	*Ca.* 5	*Ca.* 1	*Ca.* 1	0
C. .0002 M acid *plus* .0002 M Na salicylate	0	<1	*Ca.*10	35–45	25–35	*Ca.*10	*Ca.*10	*Ca.*10	*Ca.* 5	0	0	0
D. .0002 M acid *plus* .0003 M Na salicylate	<1	*Ca.* 5	25–35	15–20	*Ca.*10	*Ca.*10	*Ca.* 5	*Ca.* 1	0	0	0	0

solution of the series. Apart from this discrepancy, which can be remedied by assigning a higher value (*ca.* 2×10^{-3}) to the dissociation constant, the agreement between the observed and calculated rates of activation is satisfactory. Curve IV in Fig. 1 shows the variation in the observed optimum exposures in the different mixtures; in Curve III the calculated exposures are plotted, assuming that they are proportional to the reciprocals of the concentrations of undissociated acid (Column 7 of Table XV).

TABLE XIV.

Salicylic Acid Plus Na Salicylate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ											
	2	4	6	8	10	12	14	16	18	20	22	24
A. .0002 M salicylic acid (alone)	0	0	*Ca.* 1	15–20	30–35	*Ca.* 50	50–60	25–30	15–20	2–4	<1	0
B. .0002 acid *plus* .0008 M Na salicylate	15–20	15–20	<5	0	0	0	0	0	0	0	0	0
C. .0002 M acid *plus* .0016 M Na salicylate	25–30	<5	0	0	0	0	0	0	0	0	0	0

TABLE XV.

Activation by .0002 M Salicylic Acid in Presence of Na Salicylate.

Concentration of Na salicylate	Durations of optimum exposures (*ca.*) (T)	Relative rates of activation $\left(\frac{15}{T} \times 100\right)$	Acid dissociated (γ)	Acid undissociated ($100-\gamma$)	Relative concentrations undissociated acid $\frac{(100-\gamma)100}{14}$	Reciprocals of concentrations undissociated acid $\frac{14}{(100-\gamma)100} \times 10^4$
M	*min.*		*per cent*	*per cent*		
0	14–16 (av. 15)	100	86	14	100	100
.0001	10–12	136	82.5	17.5	125	80
.0002	8–10	167	78	22	157	64
.0003	*Ca.* 6	250	75.5	24.5	175	57
.0004	5–6	272	72.5	27.5	196	51
.0008	3–4	428	62.5	37.5	268	37+
.0016	2–3	600	48	52	372	27

General Relations of External Acidity to Physiological Activity.

The conditions under which the starfish egg is activated by acids are probably not peculiar to this cell, and in fact the foregoing observations show certain significant parallels with the conditions found in other living systems which respond definitely to variations of external acidity. For example, in the vertebrate respiratory center increase in the rate of physiological activity is also known definitely to follow increase in the external concentration of penetrating acids. Under the usual conditions, increase in the external C_H accelerates the respiratory rhythm; yet we often find experimentally a similar increase of activity even when the change of external C_H is absent or in the opposite direction.[10] Such facts have recently led to a modified conception of the relation of acidity to the respiratory rhythm. Although this rhythm is still regarded as controlled by variations of H ion concentration, it seems necessary to conclude that the essential factor to be considered is not the C_H of the external medium, but that existing within the cells of the center themselves. This, however, is only indirectly dependent on the external C_H. Jacobs[11] has recently furnished clear demonstration that the C_H of the cell interior may vary widely from that of the surrounding medium, especially when the latter contains the salts of weak acids or bases. What is true of the various cells where this demonstration can be made is in all likelihood also true of the nerve cells of the respiratory center. We may assume that these cells, possibly deficient in buffering compounds, are specially sensitive to variations of internal C_H, and vary their rate of activity correspondingly.

In the starfish egg we find conditions which in many respects are closely analogous with those just considered. In pure solutions of the activating acid increase in acidity is followed by a closely proportional increase in the rate of activation. From this fact, considered alone, we might conclude that the rate of activation is directly determined by the external C_H. But in the presence of the salts of the acid, although the relation of the rate of activation to the total concentration of added free acid is the same as before, we find a complete independence of external C_H.[1] In fact, solutions containing a fixed concentration of free acid and varying quantities of Na salt show a precisely contrary relation; increase in the rate of activation is then correlated not with increase but with decrease of external acidity. The two cases, however, are easily reconciled if we recognize that activation is dependent on the penetration not of the ions but of the undissociated molecules of the acid. These furnish the activating ions by their dissociation within the egg.

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[10] For a recent discussion *cf.* Gesell, R., *Physiol. Rev.*, 1925, v, 551; *cf.* p. 552 *et seq.* The effect just named is well illustrated in Loevenhart's work on the stimulation of the respiratory center by cyanide; *cf.* Gasser, H. S., and Loevenhart, A. S., *J. Pharmacol. and Exp. Therap.*, 1914, v, 239.

[11] Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457.

Apparently only the undissociated molecules of acid penetrate freely into the egg; there is increasing evidence that this condition is widespread among living cells.[12] In cells having this type of permeability variations of intracellular acidity, with corresponding variations in physiological activity, would tend to follow variations in the concentration of the undissociated molecules of acid or base in the external medium. This consideration explains why in the starfish egg a direct correlation between external C_H and rate of activation is found only under those conditions in which the concentrations of undissociated molecules and of H ions are also directly correlated, as in the pure solutions of the acid. In solutions containing the salt of the acid the correlation between C_H and undissociated acid may be an inverse one, as in the cases considered above: we then observe the rate of activation to follow the concentration of the undissociated molecules. External acidity is thus, taken by itself, an uncertain and variable index of intracellular acidity; it is, however, the latter which controls the rate of the physiological process.

Such a view implies that the production of acid within the cell would have the same physiological effect as the penetration of acid molecules from without. Apparently this is what occurs in the heat activation of the starfish egg. In the case of the respiratory center, Gesell has recently upheld strongly on both theoretical and experimental grounds the view that the intracellular production of lactic acid is the essential factor in the increase of respiration accompanying asphyxia.[10,13] It is clear that in considering the problem of the relation of H ions to cellular activity we must take into account not only the permeability of the cell to acids, and other features such as the buffering capacity of its protoplasm, but also the nature and quantity of the acids produced in its special type of metabolism under varying conditions.[14]

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[12] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–27, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255. Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 234. Irwin, M., *J. Gen. Physiol.*, 1926, ix, 561. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347; 1926, lxxvi, 411.

SUMMARY.

1. Comparison of the rates of activation of unfertilized starfish eggs in pure solutions of a variety of parthenogenetically effective organic acids (fatty acids, carbonic acid, benzoic and salicylic acids, chloro- and nitrobenzoic acids) shows that solutions which activate the eggs at the same rate, although widely different in molecular concentration, tend to be closely similar in C_H. The dissociation constants of these acids range from 3.2×10^{-7} to 1.32×10^{-3}.

2. In the case of each of the fourteen acids showing parthenogenetic action the rate of activation (within the favorable range of concentration) proved nearly proportional to the concentration of acid. The estimated C_H of solutions exhibiting an optimum action with exposures of 10 minutes (at 20°) lay typically between 1.1×10^{-4} M and 2.1×10^{-4} M (pH = 3.7–3.96), and in most cases between 1.6×10^{-4} M and 2.1×10^{-4} M (pH = 3.7–3.8). Formic acid ($C_H = 4.2 \times 10^{-4}$ M) and *o*-chlorobenzoic acid ($C_H = 3.5 \times 10^{-4}$ M) are exceptions; *o*-nitrobenzoic acid is ineffective, apparently because of slow penetration.

3. Activation is not dependent on the penetration of H ions into the egg from without, as is shown by the effects following the addition of

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[13] Gesell, R., *Science*, 1926, lxiii, 58; *Am. J. Physiol.*, 1923, lxvi, 5. McGinty, D. A., and Gesell, R., *Am. J. Physiol.*, 1925–26, lxxv, 70.

[14] Warburg and his associates have recently shown that various types of carcinoma cell are characterized by an unusually high production of lactic acid, under both anaerobic and aerobic conditions (Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309). They infer that the transformation of the normal resting tissue cell into the rapidly proliferating cancer cell is dependent on a change from the normal metabolism to an acid-producing or glycolytic type. The accumulation of acid within the cell would thus be the condition activating cell division in tumor-producing tissue cells as well as in the starfish egg.

its Na salt to the solution of the activating acid (acetic, benzoic, salicylic). The rate of activation is *increased* by such addition, to a degree indicating that the parthenogenetically effective component of the external solution is the undissociated free acid. Apparently the undissociated molecules alone penetrate the egg freely. It is assumed that, having penetrated, they dissociate in the interior of the egg, furnishing there the H ions which effect activation.

4. Attention is drawn to certain parallels between the physiological conditions controlling activation in the starfish egg and in the vertebrate respiratory center.

A RELATIONSHIP BETWEEN CIRCUMFERENCE AND WEIGHT IN TREES AND ITS BEARING ON BRANCHING ANGLES.

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(Accepted for publication, February 21, 1927.)

When a tree, at a point where the circumference is c_0, divides into two branches (c_1 and c_2), what relationship exists between c_0 and $c_1 + c_2$? In order to answer this question, which has a definite bearing on problems of tree form, it is convenient to investigate first the relationship between the circumference at some point and the weight, w, of all the parts of the tree peripheral to this point.

Accordingly measurements were made, 116 in all, on nine kinds of trees; namely, aspen, bitternut, hickory, oak, ash, maple, cedar, hornbeam, and beech. The largest tree measured had a circumference of 56.4 cm. where cut, and the whole tree weighed 120 kg. The smallest measurements were made on the stems of leaves,—for example, circumference of stem = 0.25 cm., weight of leaf = 0.18 gm. All the data thus obtained are included in Fig. 1.

Our procedure was of the simplest character. Whole trees of varying size, or branches, or leaves, were taken entirely at random from the vicinity. The only criterion of selection was that the specimen should not appear to have been recently injured. The circumference was measured by a tape, encircling the bark, at the point of section; or, for small specimens, the diameter was measured by calipers and the circumference subsequently calculated. The specimen was then weighed on one of three balances according to size. The season was midsummer, 1926; the place, Grindstone Island, N. Y.

Plotting logarithms; *i.e.*, log (weight in gm.) *vs.* log (circumference in cm.), the points fall close to a straight line. A statistical treatment yields the following numerical characteristics:

Mean value of log c in the observations = 0.161
" " " log w " " = 1.250

Standard deviation of log c = 0.507
" " " log w = 1.263
Mean product of simultaneous deviations = 0.637
Correlation coefficient = 0.99

From these figures one obtains, for the best linear relation between log w and log c, the equation:

$$\log w = 2.49 \log c + 0.850; \text{ or, } \qquad w = 7.08\, c^{2.49} \qquad (1)$$

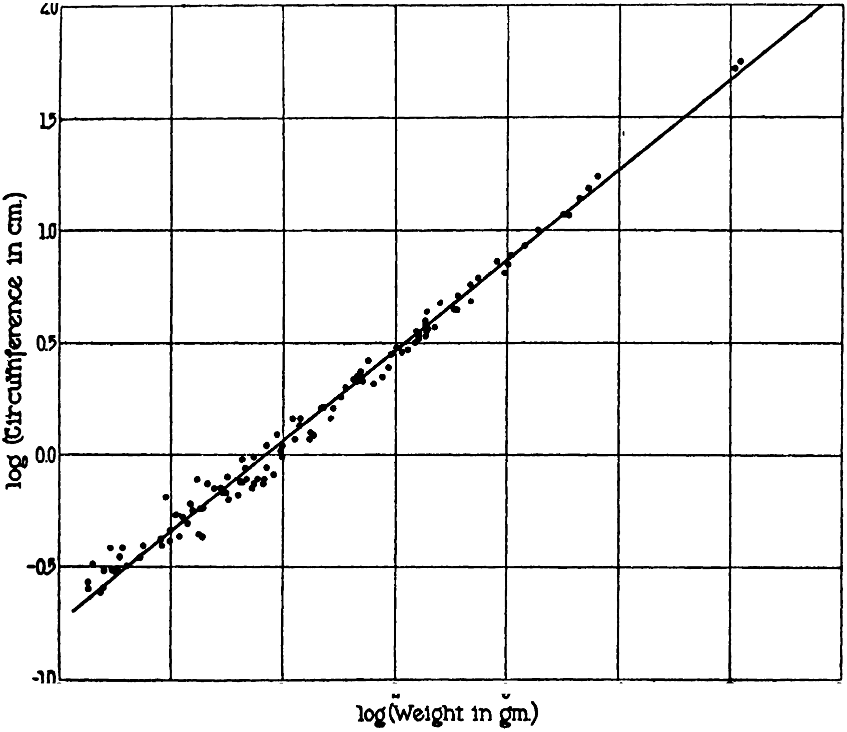

FIG. 1. The line is drawn according to equation (2). The trend toward a cube law relation among the observations appearing in the lower left portion of the chart may be significant.

The probable error of log w is ± 0.08,—*i.e.*, if from the measured circumferences estimates of the weight are calculated by equation (1), then half of the actual observed weights fall within the limits +20 per cent and −17 per cent of the calculated values. It will be seen from Fig. 1 that the error is greater for the small pieces, and less for the large pieces. We observed also that deviations from equation (1) occurring in various parts of the *same* individual tree are sufficiently large to mask any systematic differences between the different *kinds* of trees that were studied. Furthermore, the equation holds as well for stems bearing nuts as for stems bearing leaves.

Returning to the opening question, the solution, inherent in equation (1), is given by the relation:

$$c_0^{2.49} = c_1^{2.49} + c_2^{2.49} \qquad (2)^*$$

This follows from the fact that, if a main stem or trunk is cut near a point of branching and weighed (w_0), and then if the branches are weighed separately (w_1 and w_2), w_0 must equal $w_1 + w_2$.

Equation (2) describes, for the class of trees studied, one special characteristic of branching. The exponent 2.49, being greater than 2, indicates, for example, that the total cross-sectional area of the branches becomes progressively greater at each branching. To express this property we may say that trees follow statistically a "2.5 power law of branching."

Another characteristic of branching is the equation which describes the angles of branching. In a previous paper[1] this problem was discussed in reference to the *arterial* system in animals, and the following equations, of which two only are independent, were deduced:

$$\cos x = \frac{c_0^4 + c_1^4 - c_2^4}{2\,c_0^2\,c_1^2};\ \cos y = \frac{c_0^4 - c_1^4 + c_2^4}{2\,c_0^2\,c_1^2};\ \cos(x+y) = \frac{c_0^4 - c_1^4 - c_2^4}{2\,c_1^2\,c_2^2} \qquad (3)$$

where c_0, c_1, and c_2 are the circumferences of the main stem and the two branches into which it divides; and where x and y are the angles made by the branches (c_1 and c_2) with the line of direction of the stem. The angle $(x + y)$ is, of course, the angle included between the two branches. These equations, for our present purpose, may be considered as being deduced from the assumption that the branching system connecting three points shall, for given circumferences of the stem and branches, require the least volume of wood.

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*Once obtained, this relation may be roughly checked by simple measurement, without weighing or cutting, on large trees.

[1] The physiological principle of minimum work applied to the angle of branching of arteries, Murray, C. D., *J. Gen. Physiol.*, 1925–26, ix, 835.

If now equations (2) and (3) are combined one can, at the expense of loss of generality, solve directly for the angle as a function of some convenient ratio such as c_1/c_0 or c_1/c_2. The steps are shown in the previous paper. But in that paper, instead of a 2.5 power law (equation (2)), a cube law (theoretically deduced for the arterial system) was used. In either case certain qualitative rules hold which describe in words the variations in the angles accompanying variations in the ratio c_1/c_2, etc. An interesting and convenient illustration of these rules may be seen in the branching of the veins of leaves. There remains only to be observed the fact that, in changing from a cube law to a 2.5 power law, the calculated angles, for any given ratio of circumferences, become smaller,—a fact corresponding to a difference between the branching of arteries and of trees. For example, solving equation (3) for the angle $(x + y)$ when $c_1/c_2 = 1$, we find for a cube law angle $(x + y) = 75°$, for a 2.5 power law angle $(x + y) = 59°$. The curve in Fig. 2 shows this relation.

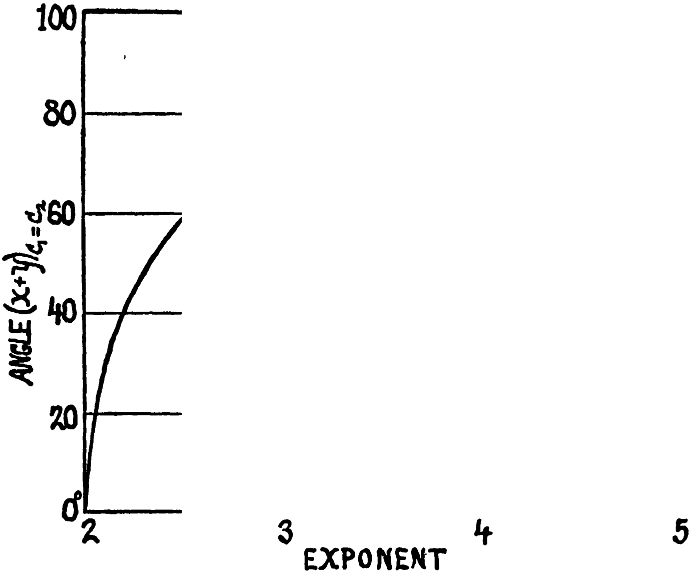

FIG. 2. The curve shows the relation between the *angle* $(x + y)$, given that $c_1 = c_2$, calculated by equations (2) and (3), and the *exponent*, considered variable, occurring in equation (2).

SUMMARY.

Observation reveals a linear relationship between the logarithm of the circumference of a tree, branch, or leaf stem, and the logarithm of the weight of the tree, branch, or leaf. The bearing of this on the angles of branching in trees is discussed.

MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

III. THE ACTION OF CO_2 AND SOME SALTS OF NA, CA, AND K ON THE PROTOPLASM OF AMŒBA DUBIA.*

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(Accepted for publication, January 28, 1927.)

The first paper (1) of this series described the action of the chlorides of Na, K, Ca, and Mg on the protoplasm of *Amœba dubia* as determined by micrurgical technique. A study of the effect of other salts of some of these cations forms the basis of this report.[1] Among those tested were a few which are often employed in preparing buffer solutions and some of general physiological interest, particularly solutions containing phosphate, borate, lactate, acetate, bicarbonate, carbonate, and CO_2. The details of the apparatus, its manipulation, and the terminology used have been fully described in a previous publication (1) to which the reader is referred.

Immersion Experiments.—When amebæ are immersed in toxic concentrations of any of the Na salts used in these experiments, the same effect is obtained as that found with NaCl (1), *viz.*, rounding, quiescence, and sinking of the heavier granules. In general, the toxicity of the phosphates depends upon the relative amount of Na in the salt (Table I). The only exception to this is the marked toxicity of NaH_2PO_4 in concentrated solutions due to the acidity of this salt (1). Alkalinity alone, as has been shown previously (1), is not a factor in the production of toxic effects. The phosphate ion may be a factor in contributing to the toxicity of these salts, since even in

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*The ameba that has been used in the studies of this series previously identified as *Amœba proteus* is the form described by Schaeffer in his book on Amœboid movement (1920) as *Amœba dubia.*

[1] We wish to thank Mr. Kenneth Blanchard for his help in preparing and analyzing some of the chemicals used in this work.

the more dilute solutions amebæ do not appear as healthy as in control solutions containing an equivalent amount of Na.

The borate is more toxic than any other salt used in this series (Table I), a condition which is in keeping with the long established use of borates as an antiseptic.

$NaHCO_3$ and Na_2CO_3 are very toxic within the first 24 hours of immersion (Table I). Even in dilute solutions, ranging from M/1024 to M/8192, the amebæ become round and sluggish. In a few hours, however, those which have retained intact plasmalemmæ recover and remain living and well. Fresh amebæ placed in these solutions which have stood for several hours are not affected. This fact indicates that a change takes place in solutions of Na_2CO_3 and $NaHCO_3$ on standing and the implication is that the loss of CO_2 is a factor in the change. To determine this, amebæ were immersed in water saturated with CO_2. Such a solution, tested immediately after preparation, had a pH of 4.8. The amebæ showed the same effects as in the carbonates. With the gradual increase in alkalinity due to the loss of CO_2, those amebæ recover whose plasmalemmæ have remained intact.

TABLE I.

Viability of Amebæ Immersed in Decreasing Concentrations of Salts.

Salts	Dead in						Living through 5 days
	1 hr.	1 day	2 days	3 days	4 days	5 days	
Monosodium phosphate..........	M/28	M/512	—	—	—	—	M/2048
Disodium phosphate.............	M/24	M/1024	—	—	—	—	M/2048
Trisodium phosphate............	M/384	M/1536	—	—	—	—	M/2048
Sodium borate..................	M/768	M/1536	—	—	—	—	M/3072
Sodium bicarbonate.............	M/256	M/512	—	—	—	—	M/1024
Sodium carbonate...............	M/384	—	—	—	—	—	M/1024
Sodium lactate.................	M/4	M/32	—	—	M/384	—	M/1024
Sodium acetate.................	M/4	M/8	M/32	M/128	M/256	—	M/512
Calcium acetate................	M/2	M/16	—	—	—	—	M/48
Calcium lactate................	—	M/7.5	—	M/15	—	—	M/30

The lactate of Na, although not markedly toxic (Table I), can inhibit activity even in very dilute solutions.

The acetate is the least toxic of all the Na salts used (Table I).

Ca acetate and Ca lactate resemble $CaCl_2$ (1) in their non-toxicity

(Table I). It is interesting to note, however, that the lactate is the only salt of Ca which has a destructive action on the plasmalemma.

Injection Experiments.—In general the injection of the Na salts produces the same effect as that of the chloride (1). With NaH_2PO_4, however, this is neither marked nor sustained. The injection of this salt is characterized by an elevation of the plasmalemma with the appearance of a subjacent hyaline zone and the formation of a distinct membrane-like film or boundary around the granuloplasm within the hyaline zone. This boundary breaks down during recovery when flowing movements of the granuloplasm fill the hyaline zone with granules, Fig. 1. Na_2HPO_4 and Na_3PO_4 are more toxic than NaH_2PO_4 (Table II), as is to be expected because of their increased Na content. These salts do not form the granuloplasmic boundary peculiar to NaH_2PO_4.

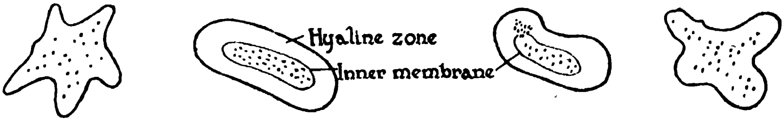

FIG. 1. The production of an inner membrane upon injection of NaH_2PO_4 into an ameba.

The borate is much more toxic than the phosphates to the interior of the ameba (Table II).

$NaHCO_3$ and Na_2CO_3 are more toxic than the other Na salts except borate because of their marked solvent action on the plasmalemma (Table II). To determine the rôle of the carbonate alone in producing this effect, bubbles of CO_2 gas were introduced into the ameba. In the cytoplasm they shrink, apparently by going into solution. If the injected bubble is larger in size than that of the nucleus, the plasmalemma fades and disappears over the entire ameba and the granuloplasm scatters. When a very small bubble of CO_2 is injected into the middle région, the plasmalemma disappears only at one end, usually the hind end of the ameba and the ameba recovers. The shrinking bubble tends to disappear before the surface breaks, Fig. 2. Control injections of bubbles of air do not affect the ameba unless the bubble is large enough to burst the ameba.

TABLE II.

Recovery of Amebæ from Injection of Decreasing Concentrations of Salts.

Salts	Recovery from			Water effect
	Small injection	Moderate injection	Large injection	
Monosodium phosphate	M/2	M/4	M/12	M/1024
Disodium phosphate	M/4	M/16	M/32	M/1280
Trisodium phosphate	M/4	M/16	M/64	M/768
Sodium borate	M/16	M/32	M/64	M/512
Sodium bicarbonate	M/16	M/32	M/64	M/256
Sodium carbonate	M/16	M/32	M/64	M/256
Sodium lactate	–	M	M/4	M/2048
Sodium acetate	M	M/3	M/8	M/128

Concentration	Pinching off effect	
	Calcium lactate	Calcium acetate
M/1	–	In 20 sec.
M/60	1 min.	–
M/120–M/130	Attempted only	In 1–2 min.
M/240–M/260	–	Delayed or attempted only
M/480	No attempt	–
M/520	–	No attempt

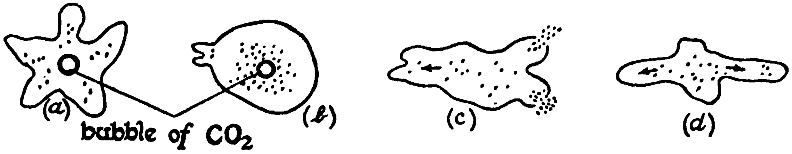

FIG. 2. The effect of injecting a small bubble of CO_2 into an ameba: (*a*) immediately after injection; (*b*) beginning rounding of ameba and sinking of granules, shrinking of bubble; (*c*) disappearance of bubble, beginning flow of ameba, breaks in plasmalemma; (*d*) recovery.

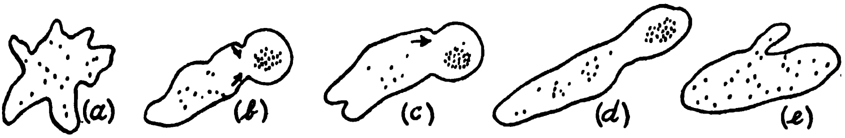

FIG. 3. The antagonistic effect of lactate on the pinching off reaction of Ca: (*a*) before injection; (*b*) beginning pinching off of solidified area; (*c*) inflow into injected area from healthy portion pushing back sluggish, constricting membrane of the stalk; (*d*) beginning incorporation; (*e*) recovery.

The lactate of Na, although relatively non-toxic (Table II), has a distinct quieting effect on the movement of the ameba and a solvent action on the plasmalemma.

The acetate is the least toxic of all the Na salts tested in this series.

Both Ca acetate and lactate have the same action as $CaCl_2$ (1), *viz.*, a solidification of the injected area which is pinched off by the living remnant. The rate of pinching off with the lactate, however, is slower than that with the acetate. This delay seems to be related to the visible sluggishness of the plasmalemma produced by the lactate. The pinching off process is frequently interrupted by an inrush of internal protoplasm. This frequently results in a failure to pinch off and, ultimately, in a consequent incorporation of the solidified material even with relatively concentrated solutions of Ca, Fig. 3.

In general, the effects of injecting the K salts were the same as those obtained with the Na salts except for the greater stability exhibited by the plasmalemma (1).

DISCUSSION.

It is evident that the predominant action of the salts is that of the cation. The anion may modify this effect without apparently changing its fundamental nature. Many of the salts tested are generally used as buffers. The usual strength of a buffer solution (M/20) is non-toxic when injected into the ameba. In immersion work, however, the buffer salts are too toxic to be used except in very dilute concentrations.

Some of the salts show individual peculiarities which are of interest. For equivalent concentrations of Na, the phosphates are more toxic in immersion experiments than the chloride. The probability that phosphates penetrate very slightly (2) suggests that their toxicity may be due to the extraction of substances, for example, Ca, from the ameba. A remarkable effect is the production of a membrane-like structure around the granuloplasm within the ameba when the acid phosphate is injected. This is perhaps due to a gelation or precipitation of some substance on the surface of the granuloplasm by virtue of an interaction with the injected phosphate. Whether the phosphate reacts with the Ca in the protoplasm it is not certain but in connection with this occurrence it is interesting to recall that an acid medium tends to accentuate the typical solidifying action of Ca (1).

The action of CO_2 and the carbonates in dissolving the plasmalemma is most significant because of the fact that CO_2 is more soluble in organic solvents than in aqueous solutions (3). This is evidence for the lipoid nature of the plasmalemma. Lillie (4) has shown in his work on the activation of starfish eggs by acids that CO_2 behaves like a fatty acid. However, the marked toxicity of CO_2 (5–7), has usually been attributed to its great penetrating power, thus implying that CO_2 exerts its toxic action on the interior. In fact, Jacobs (8) shows that CO_2 can enter and leave the cell with complete reversibility. This varies considerably with the cell used (9). The injection experiments indicate that in the ameba penetration into the interior is not *the important factor in the production of lethal effects.* In the ameba *the ability to revert to normal depends upon the maintenance of an intact plasmalemma. The ameba is irreversibly injured only when* CO_2 destroys the plasma membrane. *This emphasizes again the importance* of the surface in the maintenance of the life of the cell (10).

The lactates also act on the surface of the cell. Amebæ, immersed in these salts, are characterized by their sluggish plasmalemmæ. This is of interest in connection with Lillie's finding that lactic acid is relatively ineffective in activating the starfish egg, a condition which he attributes to difficulty of penetration. The dispersive action of the lactate on the plasmalemma is most evident when this salt is brought by injection into contact with the inside of the plasmalemma. This dispersing effect is further seen when amebæ are immersed in Ca lactate. This is the only Ca salt in which dead amebæ show a disrupted plasmalemma.

The relative non-toxicity of sodium acetate is rather surprising when one considers the frequent reports of its marked activity (4, 11). Loeb (12), however, pointed out that sodium acetate acts exactly like the chloride in its depressing effect on the viscosity of gelatin if the pH is kept constant. Furthermore, it is difficult to compare results from different materials or even different functions of the same material. Cohen and Clark, for example, point out that the effect of pH upon specific fermentative processes, upon reproduction in its several stages, and upon death, must be kept distinct. This may well be kept in mind in dealing with any factor.

CONCLUSIONS.

I. *Plasmalemma.*

1. Of the salts used in these experiments the anions have only a modifying effect on the cations. The dispersive action of Na and, to a lesser extent, of K, predominates. Borate increases the toxicity of Na and acetate decreases it.

2. CO_2 and carbonates dissolve the plasmalemma readily.

3. Na lactate tends to dissolve the surface especially when brought into contact with it from the interior by injection.

Lactate antagonizes the stimulating effect of Ca on the plasmalemma.

II. *The Internal Protoplasm.*

4. Acid phosphate of Na and K, when injected, causes a membrane to form around the granular endoplasm within the ameba.

5. Na borate increases the toxicity of Na inside the cell.

6. Bubbles of CO_2, injected into the cell, cause an increase of fluidity of the internal protoplasm. These bubbles shrink and disappear from the cell more readily than air bubbles.

7. The anions modify the typical cation effect. Carbonates accentuate the liquefying and solvent action of Na.

Phosphates prevent a complete rounding of the ameba caused by Na.

Lactate inhibits the solidification and pinching off effect caused by Ca.

III. *Physiological Significance of Salts.*

8. The buffer salts can be injected in high concentrations without toxic effects but amebæ can be immersed in them only in very dilute solutions without injury.

9. The inhibiting action of lactate and the dispersive effect of CO_2, carbonates, and lactate on the plasma membrane, must be of importance in a consideration of the functions of the organism and perhaps in the production of pathological changes.

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MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

IV. COLORIMETRIC DETERMINATION OF THE NUCLEAR AND CYTOPLASMIC pH IN THE STARFISH EGG.*

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(Accepted for publication, February 16, 1927.)

An essential feature in determining the hydrogen ion concentration of protoplasm is the maintenance of a normal condition of the protoplasm during the procedure. Results obtained by immersing cells in solutions of dyes have been inadequate owing to the lack of satisfactory indicators to which living cells are freely permeable (1). Attempts have been made to overcome this difficulty by artificially altering the permeability of living cells (2). Any procedure, however, which exposes the cells to abnormal conditions may seriously affect the results obtained. The existence of natural dyes in the tissues has been utilized (3, 4) but has not given definite results. Some investigators have made both potentiometric and colorimetric determinations of cellular extracts (5, 6).

Recently, Vlès and his coworkers (7–10) have introduced a method (méthode microscopique d'écrasement) by means of which echinoderm eggs, immersed in an indicator solution, are carefully crushed between the cover slip and slide of a compressorium. As soon as the egg bursts pressure is released whereupon the dye passes in through the breaks over the surface of the egg. The objection that the pH of crushed cells may be quite different from that of the living protoplasm has already been considered by the Needhams (11). The results obtained by the micrurgical technique have brought out the importance of the plasma membrane for the maintenance of protoplasm (12–14). If a cell is crushed so that the plasma membrane disintegrates, the exuding material either merges with the environing medium and is destroyed or forms spherules of normal appearing protoplasm walled off by new surface membranes. From experiments described in this paper these spherules are no more permeable than the protoplasm in its original condition. The disintegrated material scatters or rounds up into droplets which swell and burst.

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A potentiometric determination of the pH with the aid of the micrurgical apparatus has been made possible recently by the development of the micro electrodes of Ettisch and Péterfi (15) and Taylor (16). However, the use of micro electrodes thus far has not met with success in the determination of the pH of the protoplasm.

The micro injection of indicator dyes into the protoplasm of cells has met with considerable success. Kite (17) was probably the first to inject dyes into the protoplasm of a cell. With a meagre supply of indicators at his disposal he concluded that the interior of the ameba is faintly alkaline. More recently Schmidtmann (18, 19) introduced solid particles of dyes into mammalian tissue cells. He obtained values of pH varying from 5.9 to 7.8 in different cells. The Needhams used aqueous solutions of the Clark and Lubs series of indicator dyes and determined the internal pH of a number of marine ova to be 6.6 ± 0.1.

The investigations described in this paper were carried out principally because of the discrepancy between the results obtained by the Needhams and those of other investigators—notably Vlès, Reiss, and Vellinger (7–10)—and because of the desirability of also determining the pH of the nucleus.

A. Methods and Material.

The experiments were performed on the eggs of the echinoderm *Asterias forbesii.* The eggs to be injected with indicator solutions were immersed in hanging drops of normal or of acidified sea water. The pH of normal sea water when determined colorimetrically is 8.4 and, potentiometrically, 8.2 (20). To obtain acid sea water, KH_2PO_4 was added.

The dyes used were neutral red and those of the Clark and Lubs series of pH indicators covering the range from 4.4 to 8.4 (Sörensen units), *viz.*, methyl red, brom cresol purple,[1] brom thymol blue, phenol red, and cresol red. The neutral red was made up in a saturated aqueous solution and NaOH was added until the solution changed from a red color to a deep orange red with no sign of a precipitate. All the Clark and Lubs' indicators used were obtained from Hynson, Westcott, and Dunning, Baltimore. The dyes were prepared according to Clark (20) in 0.4 per cent aqueous solution with a molecular equivalent of NaOH. The brom thymol blue was found to be decidedly toxic upon injection into the eggs. All the other dyes were relatively non-toxic except brom cresol purple which gave evidence of toxicity only when injected into the cell nucleus. The Needhams, who used dyes from the British Drug Houses, did not find brom thymol blue to be especially toxic. On the other hand, they reported that the brom cresol purple produced cytolysis with considerable ease.

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[1] A peculiar feature of brom cresol purple is that the color of its alkaline range under the microscope appears distinctly blue rather than purple.

Fortunately, all the dyes in Clark's series for determining the pH are used as sodium salts and do not cause coagulation but quickly spread through the protoplasm and give it an even, diffuse color. This feature has already been noted for certain other acid dyes (21).

Neutral red, a basic dye (either the chloride or iodide of the color base), tends to coagulate protoplasm when it is injected (13, 21). If very little is introduced, the coagulating effect is localized at the spot of puncture and the dye diffuses slowly and evenly through the rest of the protoplasm. The diffuseness disappears after some time when the color accumulates in or on the cytoplasmic granules. Regions which are thickly beset with granules then appear more deeply colored than regions where the granules are sparse. In the following series of experiments the tints were recorded while the color was still in the optically homogeneous cytoplasm.

The dyes were injected both in their alkaline and acid states and in varied quantities. As long as the injection produced no visible signs of irreversible injury to the protoplasm the color always turned to that characteristic of a constant pH value. There was, therefore, no danger of masking or swamping out the cytoplasmic pH by the possible introduction of an excessive amount of the indicator solution.

The use of a completely overlapping series of indicators which show actual changes in tint rather than intensity differences were depended upon for determining the pH. Comparisons of the colors were made with the indicators in Clark and Lubs' standard buffer solutions. Direct comparisons on the stage of the microscope were also made by means of capillary glass tubes filled with the dye and by Pantin's method (22) of projecting the image of a series of colored test-tubes into the microscopic field.

The source of illumination was a 100-Watt nitrogen-filled, tungsten (Mazda C) bulb the magnified image of which was cast on the plane mirror of the microscope by means of a glass globe filled with water. Between the globe and bulb was inserted a ground "Daylite Glass," a color screen devised by Gage (23) for producing daylight artificially.

A Leitz aplanatic-achromatic, N.A. 1.40, condenser was used with its top lens removed (24). For critical reading the Leitz, 3 mm. apochromatic objective with an 8 X, periplan ocular was used.

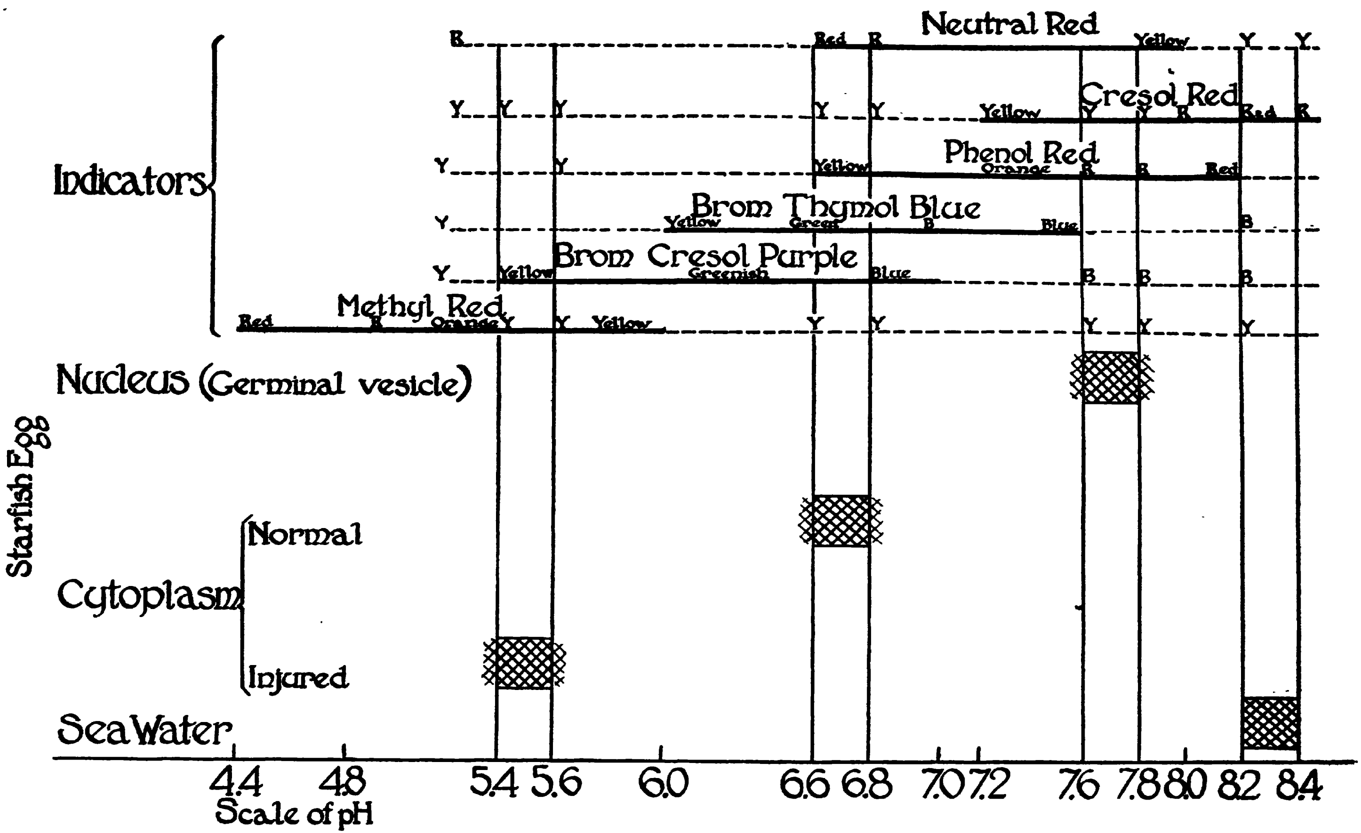

FIG. 1. Tabular representation of the pH determined for the normal and injured cytoplasm, and the interior of the germinal vesicle of the starfish egg.

B. Experiments.

1. The Cytoplasm.

The dyes, when injected into the cytoplasm of the starfish egg, give colors which indicate a pH of 6.7 ± 0.1, Fig. 1. This value was determined from the injection of phenol red which gave a yellow color with no appreciable red tinge. The true colorimetric value may be one or two decimal points above this figure owing to the fact that any tinge of red would be obscured by the faint yellow pigment present in the normal cytoplasm. The same value was obtained for the cytoplasm of eggs in the unfertilized, fertilized, and the first and second cleavage stages. Our results, therefore, closely approximate those of the Needhams.

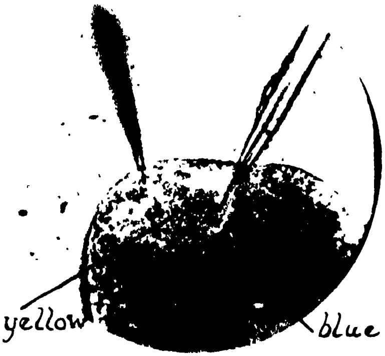

FIG. 2. Photograph of mature, unfertilized egg of the starfish injected with brom cresol purple and locally injured by a thrust of a micro needle. The region cytolyzed by the injury is yellow, the healthy cytoplasm is blue.

2. Effect of Injury on the Cytoplasm.

(a) Injury Accompanied by Visible Disintegration. A rapid tear of the cytoplasm of an egg induces cytolysis which spreads from the spot of injury (12, 13). Frequently, the spread of the cytolysis is stopped by the formation of a new membrane between the healthy and cytolyzing cytoplasm. Such a case is illustrated in Fig. 2.

An unfertilized starfish egg, colored blue by injecting brom cresol purple, was injured by repeated thrusts of a micro needle. The photograph was taken after the cytolysis had been localized. The immediate change in color of the cytolyzing region from blue to yellow shows that there is a rapid production of an acid due to injury which changes the pH to 5.6 or lower. A similar treatment of an egg colored yellow with injected methyl red results in no color change. This shows, *cf.* Fig. 1, that the pH of cytolysis is between 5.4 and 5.6. The same value was obtained when eggs were cytolyzed in sea water as acid as is consistent with viable conditions (pH 6.0). The cytolyzed region keeps its pH for several minutes until the seeping in of the sea water shifts the pH to that of the surrounding medium.

A phenomenon which may be of significance in a study of cytolysis occurs if a starfish egg, injected with brom cresol purple, is injured so as to produce extensive cytolysis. The yellow, disintegrating material gradually separates into two constituents: a loose, granular coagulum, colored yellow, and an oil-like, free flowing liquid, colored blue. On standing, the latter becomes semi solid.

The acid due to mechanical injury can also be detected in the environment of the egg. This is shown in the following experiment. An immature starfish egg, immersed in sea water colored with brom cresol purple was injured with a needle. Prior to visible cytolysis of the egg, the sea water immediately around it turned yellow and, after a few seconds, reverted to the original blue color.

(*b*) *Injury Unaccompanied by Visible Disintegration.*—The mere fact that a slight tear or puncture of an egg causes no morphological changes characteristic of cytolysis does not indicate that no injury has resulted. The two following experiments offer evidence that an acid due to injury is produced with no consequent visible cytolysis when a micro pipette punctures an egg in the course of an injection or when the egg is slowly torn. A micro pipette, having an aperture of half a micron, was filled with brom cresol purple in its blue state. The pipette was then thrust into an egg and the dye immediately injected. The region of the puncture at once took on a distinctly yellow color in contrast to the blue which slowly spread throughout the rest of the cytoplasm. 1 or 2 seconds after the injection the yellow color, at the spot where the puncture had been made, changed to a blue. In the other experiment a starfish egg, previously injected with brom cresol purple, was carefully punctured and slowly torn with a needle. A flash of yellow appeared in the immediate vicinity of the puncture quickly followed by a return to the blue.

In both of the above experiments the loss of the yellow color resulted in a disappearance of the only evidence that the cytoplasm had ever been punctured or torn.

3. The Nucleus of the Immature Egg.

The susceptibility to mechanical injury of the germinal vesicle or nucleus of the immature starfish egg has been previously demonstrated (12, 13).

By taking special precautions it was possible to insert a pipette into the germinal vesicle and to inject indicator dyes into it with no visible sign of injury. The most serviceable pipette for this purpose is one with a tip which tapers rapidly and then extends as a hollow, rigid hair 8 or 10 micra long and a little over 1 micron in diameter at its base. The aperture at the hair tip is less than half a micron in diameter. Pipettes of hard or Pyrex glass are brittle and too easily broken. Soft glass pipettes are more satisfactory and can be rendered sufficiently free of alkali for the period of the experiment by rinsing before use.

An egg was held with a micro needle against the edge of a hanging drop of sea water. The pipette was then thrust into the egg and slowly pushed against the nucleus which it indented. The tip of the pipette finally broke through the nuclear membrane without causing visible injury. After a small amount of the indicator had been injected, the pipette was slowly withdrawn and the minute puncture closed as the indentation of the nuclear membrane flattened out. The egg was then pushed into the deeper region of the hanging drop where the nucleus resumed its normal shape and appearance except for the color of the injected dye. In this way all the dyes indicated in Fig. 1 except brom thymol blue and methyl red were successfully injected. Brom thymol blue was omitted because of its toxicity and methyl red because its useful range is too low.

The colors assumed by the dyes indicate an intranuclear pH between 7.4 and 7.6 (*cf.* Fig. 1). Brom cresol purple, in addition to coloring the nuclear sap blue, fixes the nucleolus and stains it an intense purplish blue. Phenol red and neutral red are the least toxic and it was after the injection of these two dyes that a maturation of the injected germinal vesicle was observed. Fig. 3 shows three photographs of an egg whose germinal vesicle was injected with phenol red. In Fig. 3, 1, the tip of the pipette can be seen at *a* in the germinal vesicle of the egg which is flattened by being brought into the shallow part of the hanging drop. After the injection the germinal vesicle was colored diffusely rose red. Some of the dye passed into the cytoplasm either directly or through the nuclear membrane. The yellow color of the cytoplasm and the red of the nucleus offered a striking contrast. Fig. 3, 2, is a photograph of the egg 1 hour later when it had been returned to the deeper region of the hanging drop. The onset of a typical maturation is to be noted. The germinal vesicle has begun to collapse and its membrane to wrinkle and fade. The red nuclear sap streamed in several radial paths into the yellow cytoplasm which took on an everdeepening orange tint. After several minutes the orange color changed back to the original yellow. The last photograph, 3, was taken 1 hour later and shows the diminutive pronucleus in the state which precedes polar body formation. In the four cases in which this phenomenon was observed (three after the injection of phenol red and one after that of neutral red) no polar bodies were formed.

FIG. 3. Photograph of starfish egg undergoing maturation with its cytoplasm and germinal vesicle injected with phenol red. 1. Immature egg held by needle "*b*" in shallow region of hanging drop and with micro pipette "*a*" vertically inserted into the germinal vesicle. The cytoplasm is yellow, the germinal vesicle is red. 2. Egg, 1 hour later, in deeper region of hanging drop. The germinal vesicle has begun to shrivel and the cytoplasm is taking on an orange tint. 3. Egg 10 minutes later, with yellow cytoplasm. The diminutive pronucleus prior to polar body formation can be seen in the center of the egg.

FIG. 4. Sketches to show effect of mechanically injuring the germinal vesicle of a starfish egg. 1. Before injury. 2. Immediately after injuring the egg either by crushing or by puncturing the germinal vesicle. The remains of the germinal vesicle is to be seen as a hyaline sphere (nuclear remnant) and the cytoplasm around it has cytolyzed. The vitelline membrane is partially lifted, a phenomenon which frequently occurs when an egg is injured, *cf.* Chambers (12). 3. Completely cytolyzed egg within vitelline membrane.

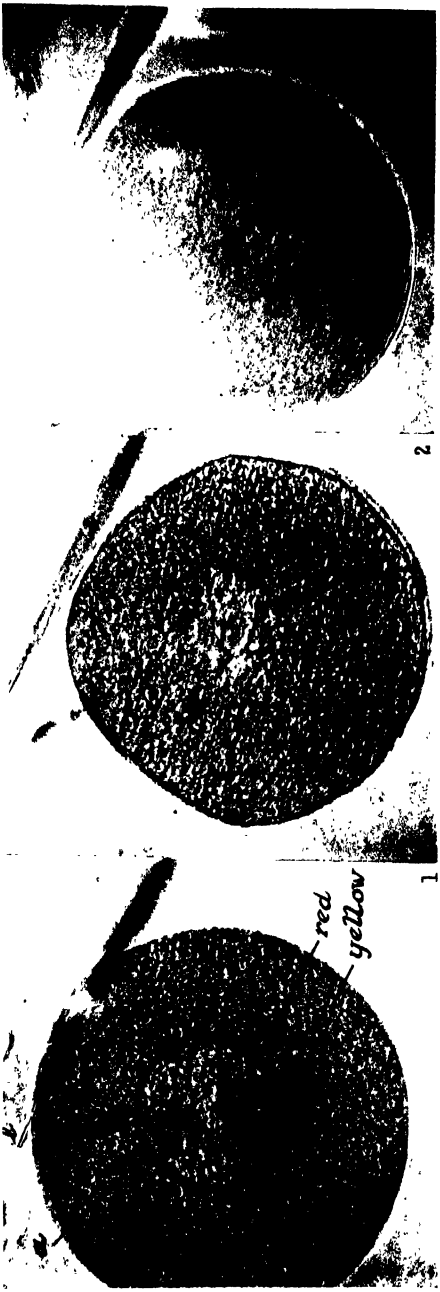

FIG. 3

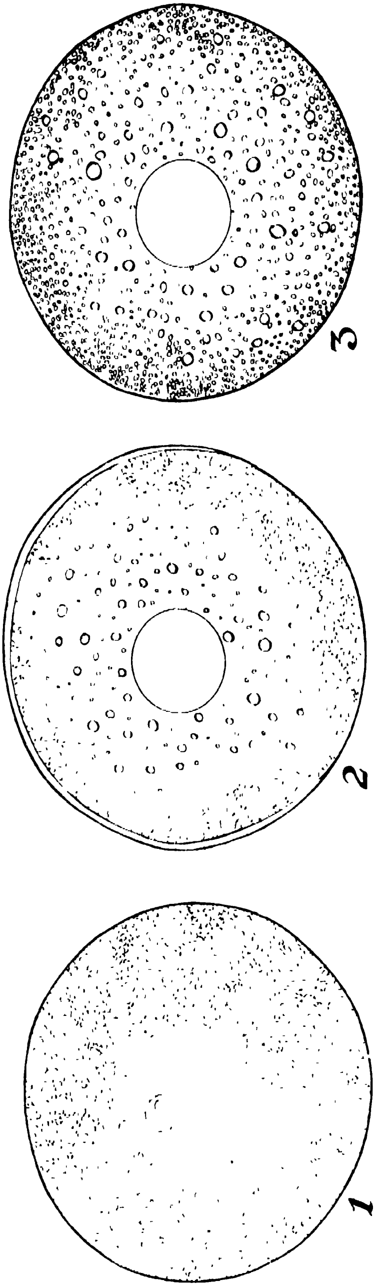

FIG. 4

The uniform orange tint in the germinal vesicle after an injection of neutral red was followed almost immediately by a deep, red zone in the bordering cytoplasm. The color in the nucleus rapidly became paler and almost completely faded while the cytoplasm tinged a rose red. Neutral red was the only dye which faded from the nucleus to such an appreciable extent.

The nucleolus is a more or less solid body and tends to become more intensely colored in time than the rest of the nucleus. When a stream of neutral red is directed against the nucleolus, the color of the dye spreads slowly through it from one side.

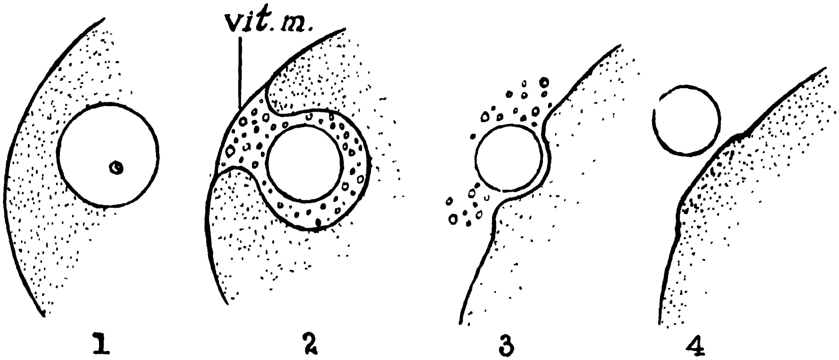

FIG. 5. Injury of germinal vesicle followed by extrusion of nuclear remnant. 1. Before injury. 2. Nuclear remnant in cytolyzed region which is walled off from healthy cytoplasm. vit. m. = vitelline membrane. 3 and 4. Nuclear remnant which is being extruded after breakdown of vitelline membrane.

4. *Effect of Injury on the Germinal Vesicle.*

The usual sign of approaching disruption is the fading of the nucleolus. This is followed by a cytolysis which spreads from the surface of the injured nucleus. A spherical, optically homogeneous, nuclear remnant frequently persists in the disintegrated region, Fig. 4, and can be dragged out into the surrounding sea water. Brom cresol purple has the peculiar property of frequently fixing both the nucleolus and the nuclear membrane without hindering disintegration of the rest of the egg.

If an injected germinal vesicle is injured there is no change in color regardless of the indicator used. This is in striking contrast to the immediate change which is produced in the cytoplasm upon injury. The germinal vesicle and cytoplasm of a starfish egg, Fig. 5, 1, were injected with phenol red and injured so as to produce cytolysis, Fig. 5, 2. The healthy cytoplasm and the débris resulting from cytolysis were yellow and the nuclear remnant red. In Fig. 5; 3 and 4, the healthy egg remnant has rounded up and the consequent flattening out of the bay has carried the nuclear remnant and the cytolyzed débris into the surrounding sea water. The débris turned red on coming into contact with the sea water the pH of which is 8.4. The nuclear remnant maintained its red color for several seconds until it collapsed and disappeared. This shows that injury to the nucleus causes no increase in acidity.

Cresol red was injected into the germinal vesicle of another egg. Upon injury the nuclear remnant retained the yellow color until it collapsed in the cytolyzed débris. This shows that injury to the nucleus causes no increase in alkalinity.

The ease with which the nuclear remnant assumes the color of its environing medium is seen from the following. If brought into sea water colored with phenol red it turns red if the sea water has a pH of 8.4 and yellow if it has a pH of 6.0.

A germinal vesicle was injected with brom cresol purple. Upon injury to the nucleus, extensive cytolysis of the egg ensued. The acid débris and the blue nuclear remnant were retained within the confines of the persisting vitelline membrane. The nuclear remnant gradually changed from a blue to a yellow color, indicating that it had assumed the pH of its immediate environment of cytolyzed material.

5. The Rate of Surface Membrane Formation in Its Relation to the Entrance of Dyes through a Torn Surface of a Starfish Egg.

Frequently, if cytolysis occurs when too large a puncture is made in injecting a plasmalemma quickly forms about the cytolyzed area and the fluid ejected from the pipette simply lies in a pocket sharply marked off from the healthy cytoplasm. When this occurs with eggs immersed in normal sea water, the newly formed membrane serves as an effective barrier against the passage of the dye into the cytoplasm. Evidently, the membrane must form with extreme rapidity. If, however, the same procedure is carried out on eggs in sea water having a pH of 6.0, the dye frequently penetrates into the cytoplasm. This indicates that this new surface membrane in an environment which is more acid than normal, either has a different permeability, or is retarded long enough in its formation to allow the dye to enter. It is possible that both factors are operative.

DISCUSSION.

Of the considerable number of papers which have recently been published on intracellular pH, only those can be specially mentioned which deal with marine ova. Our results on the pH of normal cytoplasm of starfish eggs are in close accord with those of the Needhams. We place the pH of normal cytoplasm of the eggs between 6.6 and 6.8, the Needhams place it at 6.6 ± 0.1.

TABLE I.

Differences in Dissociation of Acid and of Basic Dyes in Their Acid and Alkaline Ranges.

Dyes		Acid range	Alkaline range
Acidic	Brom cresol purple	Low dissociation Yellow	High dissociation with salt formation Blue*
	Phenol red	Low dissociation Yellow*	High dissociation with salt formation Red
Basic	Methyl red	High dissociation with salt formation Red	Low dissociation Yellow*
	Neutral red	High dissociation with salt formation Red*	Low dissociation Yellow

* Color assumed by the cytoplasm when the dye is injected into it.

In answer to the possible objection that the errors are too great to permit a determination of the protoplasmic pH, it may be pointed out that all the dyes give consistent indications toward the same pH irrespective of their chemical constitution, Table I. For example, brom cresol purple gives to the cytoplasm the blue color of the salt of its alkaline range while phenol red imparts to the cytoplasm the yellow, non-dissociated color of its acid range. The same principle also holds true for the two basic dyes used: *viz.*, neutral red and methyl red.[2] Both are yellow in their alkaline ranges where they are in the state of lowest dissociation and least salt formation. In their acid ranges, where their dissociation is greatest and, consequently, where salt formation predominates, both of the dyes are red in color. The fact that methyl red gives a yellow while neutral red a rose red color when injected into the cytoplasm is added evidence that the hydrogen ion concentration is a prime factor in the formation of the colors.

The production of an acid associated with injury or death of cellular tissues has been frequently reported in the literature (3, 4, 19, 25).

Concerning the pH of the injured cytoplasm of echinoderm eggs our results differ somewhat from that of the Needhams. In the eggs of *Paracentrotus lividus* they placed the value below 5.0 and above 4.0 because of the results obtained with methyl red and brom phenol blue. The methyl red we used was the sodium salt while they used the saturated aqueous solution.

The difference in reaction of the egg to a slow and to a rapid tear is probably due to the amount of acid produced by the injury. With a slow tear very little acid results at any given moment and it is presumably neutralized as fast as it is formed. With a rapid tear a considerable amount of acid is produced which cannot be taken care of by the cytoplasmic buffers upon which cytolysis sets in. With the spread of the disintegration more acid accumulates and the cytolysis continues. It is also significant that mechanical injury occasions an increase in acidity both outside the egg and within its cytoplasm, before there is any visible sign of cytolysis.

In this regard it is of interest to note the pH findings on echinoderm eggs by Vlès and his coworkers. They crush the eggs in the indicator and observe the resulting color. This method is open to several objections: first, there is the danger of mixing the intracellular fluids with the fluid which surrounds the cells; second, the cytoplasmic fluid may also mix with the fluids of intracellular vacuoles; and, third, the injury to the plasma membrane, which is a necessary consequence of crushing cells, almost always initiates disintegrative changes in the protoplasm. In addition to this the instantaneous production of an acid upon mechanical injury followed by the further development of acid concomitant with visible cytolysis must, to a considerable degree, modify the actual pH of normal, uninjured cytoplasm.[3]

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[2] Methyl red has both an active acid and basic group in its molecular structure. The indicator is generally used as the sodium salt, but it shows the typical dissociation curve of a base. It is the basic group which is responsible for the color changes from red to yellow.

After the publication of the Needhams' criticism of the crushing method Vellinger (27) checked the previous potentiometric determinations (9) of egg material procured by crushing the eggs in a chamber cooled to −60°C. Potentiometric readings were then made on the powder as it thawed. The first readings gave the highest pH. Subsequently, as the temperature rose, the pH dropped until it reached a constant value equal to that already recorded by Vlès, Reiss, and Vellinger (9) as the normal pH of the cytoplasm of the eggs. The fact that Vellinger's first readings give the highest pH, can be interpreted to mean that the excessively low temperature prevents or at least delays the production of the acid accompanying injury when the eggs are crushed. The first readings should then more nearly approach the pH of the normal cytoplasm. With the progressive thawing of the egg material more and more acid is produced and hence the pH falls till it reaches a level typical for cytolysis.

In this connection may be mentioned the recent result of Bodine (28) who obtained some fluid from the large yolk-laden *Fundulus* egg by pricking the dried surface of the egg. The exuding fluid was drawn into a dry glass capillary. The pH of the fluid, determined potentiometrically, was found to be 6.4.

The Needhams made no special investigation of the pH of the germinal vesicle but it is significant that they report it to give the alkaline color of brom cresol purple in both the *Echinocardium* and the *Asterias* egg even after cytolysis had occurred.

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[3] Reiss (26) claims to have found by his crushing method that the pH of the *Paracentrotus* egg changes during the different stages of its development. The values which he gives lie between the extremes of 5.3 and 5.6 and are small enough to be considered within the limits of probable error. However, it is conceivable that difference in pH may occur in the disintegrated material obtained from cells in the different stages of their development.

The fact that the nucleus does not change in reaction after cytolysis of the egg, indicates why Reiss was able to report from his results on eggs crushed in indicator solutions that the nucleus is faintly alkaline. This is in accordance with the results we obtained with the nuclear remnants of crushed *Asterias* eggs.

In order, however, to determine the pH of the normal nucleus there must be a definite proof, as given in the experimental part of this paper, that both the egg and its nucleus are alive and active during the period of the determination.

The difference in the hydrogen ion concentration between the nucleus and cytoplasm of the living immature starfish egg is of considerable interest. It would, however, be premature to speculate from this on the interrelationships of the nucleus and cytoplasm of cells in general. On the other hand, it might well be pointed out that the immature egg, although it has a much enlarged nucleus, is, nevertheless, more truly to be compared with a somatic cell than the mature egg.

SUMMARY.

I. *Cytoplasm.*

1. The normal cytoplasmic pH, colorimetrically determined, of the starfish eggs in the unfertilized, fertilized, and first and second cleavage stages is 6.7 ± 0.1.
2. Cytolysis lowers the pH to a value 5.5 ± 0.1.
3. The cytolyzed material in time assumes the pH of its environing sea water.
4. The acid due to mechanical injury can also be detected in the environment of the egg.
5. Injury to the cytoplasm unaccompanied by visible disintegration causes an increase in acidity which is quickly neutralized.

II. *Germinal Vesicle.*

6. The intranuclear pH, colorimetrically determined, of the immature *Asterias* egg is 7.5 ± 0.1.
7. Injury to the nucleus does not change its pH.
8. The spherical nuclear remnant which persists after injury gradually assumes the pH of its environment.

III. *Plasmalemma.*

9. A dye to which the cell is normally impermeable can penetrate through a tear in the surface from an environment more acid than normal. This may be due to a difference in the formation of the plasmalemma in a normal and an acid medium.

We take this opportunity of thanking Dr. Barnett Cohen of the Hygienic Laboratory, Washington, for valuable advice given in the preparation of this paper.

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GEOTROPISM OF AGRIOLIMAX.

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I.

Analysis of the geotropic conduct of young rats (Crozier and Pincus, 1926–27, *a, b;* Pincus, 1926–27) showed that it is possible to describe the negative geotropism of these animals by simple mathematical expressions. The results gotten from mammals may be shown to have a general value, as soon as it becomes possible to find similar relationships with invertebrates.

The negative geotropism of gastropods, as in *Helix* (Cole, 1925), *Limax* (Crozier and Federighi, 1924–25; Davenport and Perkins, 1897–98), and other forms (Kanda, 1916) suggested that such animals could be used for an exact investigation of their geotropic conduct. The common garden slug *Agriolimax lævis campestris* (Binney) was available during fall and winter months, and was used for these experiments.

II.

A large number of animals was kept for several weeks in the dark room, where the experiments were made. During an experiment several individuals were put on an inclined plate of ground glass, which was moistened every few minutes. In darkness the slugs creep almost always upward, and it was interesting to see whether we could find here in the same way an increase of the angle of orientation if the angle of the creeping plane with the horizontal was increased, as in the experiments with the young rats. The angle of inclination of the glass plate was varied between 45° and 90°. As soon as the animals were put on the plate they started to creep and described a certain path which was observed every few minutes with a red flash-

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The graph in Fig. 1 gives a picture of this relationship. The points fall almost altogether on a straight line, aside from the two points for the observation with $\alpha = 45°$ and $\alpha = 90°$. When the creeping plane is inclined at about 45° it seems that the total downward pull is too small, so that the lower limit of geotropic excitation is reached, and the animals begin to show frequent circus movements as during the experiments with still lower angles. In case the creeping plane is vertical, the animals seldom creep straight up; one gets always a little deviation to the right or to the left. Measuring the angles, we get up to 5° deviation from the vertical; but for simplification only angles between 85° and 90° were recorded, not between 90° and 95°; this explains why Θ, at $\alpha = 90°$, must appear to be a little less than 90°.

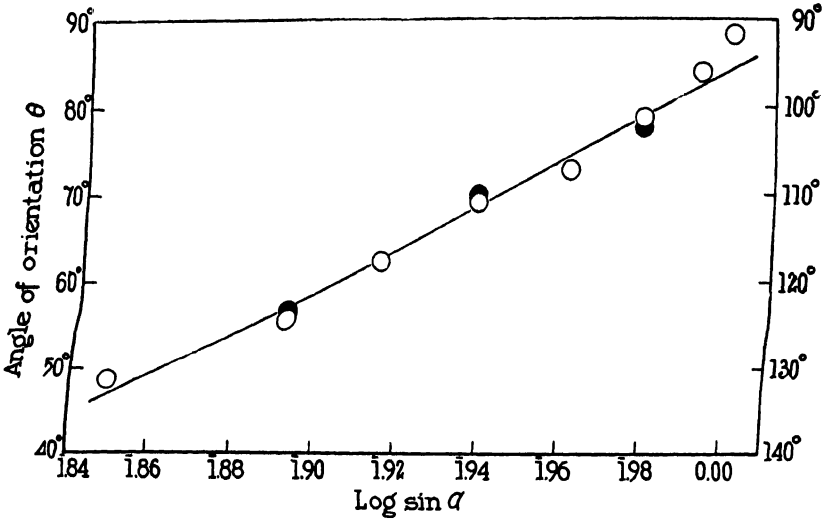

FIG. 1. The angle of orientation (Θ) is directly proportional to *log sin α*, where α is the angle of inclination of the creeping plane. The points are averages of 30 to 50 measurements each. The solid circles are from measurements made at inclinations greater than 90° (*i.e.* the animal hanging from the under surface of the plane).

The line fits the points gotten in these experiments where the animals were in the upper side of the glass plate, and fits equally well the points for the angles (α) beyond 90° where the animals were hanging from the under side of the plate.

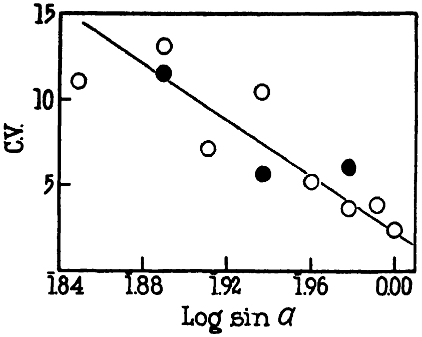

FIG. 2. The coefficient of variability of the angle of orientation decreases steadily with the increase of the logarithm of the sine of the angle of inclination of the creeping plane (see text).

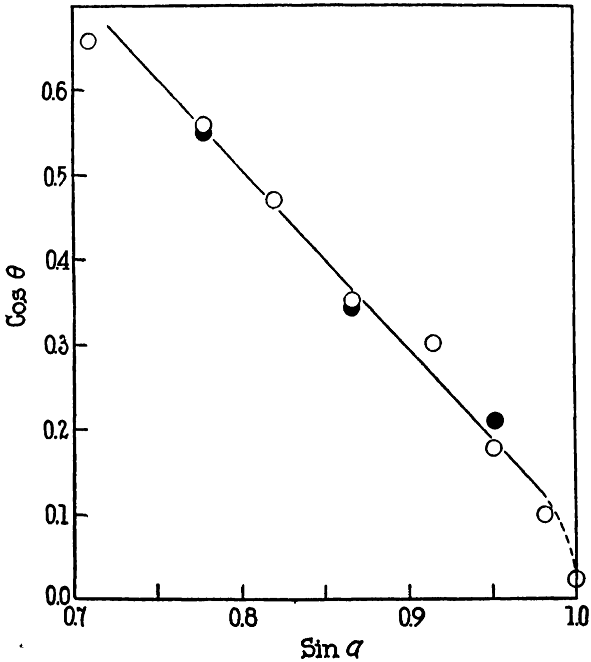

FIG. 3. The cosine of the angle of orientation (θ) decreases almost in direct proportion to the sine of the angle of inclination (α) of the creeping plane.

The coefficient of variability of the measured angles of orientation, which measures inversely the precision of orientation, decreases steadily as *log sin* α increases (Fig. 2). This corresponds to the results with rats (Crozier and Pincus, 1926–27, *a, b*). The scatter of the figures is greater than with the rat observations, probably because the animals in the present experiments form a less homogeneous population; factors unimportant for the *mean extent* of orientation may obviously influence its precision, and probably vary with the season of the year and with the size of the individual.

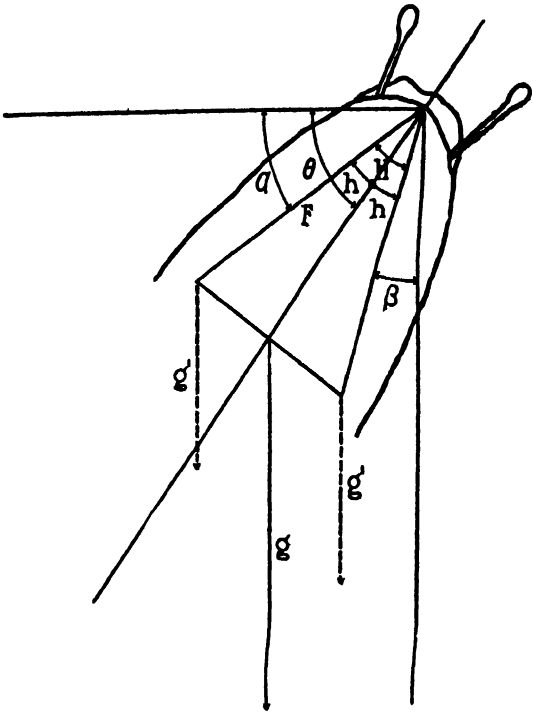

Fig. 4. Diagram showing terms used for the explanation of the relationship between the sine of the angle of orientation (Θ) and the reciprocal of the sine of the angle of inclination of the creeping plane.

III.

After having gotten the logarithmic relationship between the pull of gravity and the extent of the orienting response of the animals, the question arises, whether for the slugs, as in the experiments with the young rats, the cosine of the angle of upward orientation (Θ) decreases directly in proportion to the sine of the angle of the inclination of the creeping plane (α). Fig. 3 shows that for *Agriolimax* we apparently find the same relationship, at least quite nearly.

In the case of the rats the explanation for this was given on the basis of the different extension of the legs on the "up" and on the "down" sides of the body. The magnitude of Θ was dependent on the differential pull on the legs. In the slugs an explanation of this kind could be given by assuming that there is a difference in the pull of the weight or a difference in the stress of the muscles of the two sides of the body; but neither of these assumptions can lead to a satisfactory result, because we have in neither case the possibility to get a quantitative expression for the distribution of the weight or of the muscle tensions. Nevertheless, it can be shown that by plotting *cos* Θ against *sin* α we get a straight line which fits the points of the observation (Fig. 3). A very much better explanation of the conduct of the slugs can, however, be given in the following way.

In the slug the muscle fibers of the anterior end of the body diverge at a certain angle (H); the angle between the axis of the body and the body wall is $H/2 = h$. The angle between the body wall and the horizontal on one side of the animal $= \alpha$, on the other side the angle between the vertical and the body wall $= \beta$; furthermore, the angle of orientation (Θ) $= \alpha + h$ (Fig. 4).

On both sides of the slug the component of gravity (g) acts in the same way; when orientation is reached we may assume (*cf.* Fig. 4) that

$$g'x\,[\cos\alpha - \cos(\alpha + H)] \text{ becomes constant;}$$

$$\therefore \cos\alpha - \cos(\alpha + H) = \frac{K}{g},$$

$$\text{or } \cos(\Theta - h) - \cos(\Theta + h) = \frac{K}{g},$$

$$= \cos\Theta\cos h + \sin\Theta\sin h - \cos\Theta\cos h + \sin\Theta\sin h,$$

$$\text{and } \sin\Theta\sin h = \frac{K'}{g},$$

$$\sin\Theta\sin\alpha = \frac{K''}{\sin\,h} = \text{constant},$$

$$\therefore \sin\Theta = \frac{K''}{\sin\alpha}.$$

As shown in Fig. 5, the agreement of the observations with this equation is better than in the case of the cosine formula. The explanation here adopted, while slightly different from that used for the rats (Crozier and Pincus, 1926–27, *a*, *b*) in reality turns upon exactly the same sort of effect, namely the distribution of the pull of the animal's weight upon the two sides of the body. The fact that the cosine formula is almost obeyed (Fig. 3) is due to the fact that 1/*sin* θ is very nearly proportional, for angles not too small, to *cos* θ.

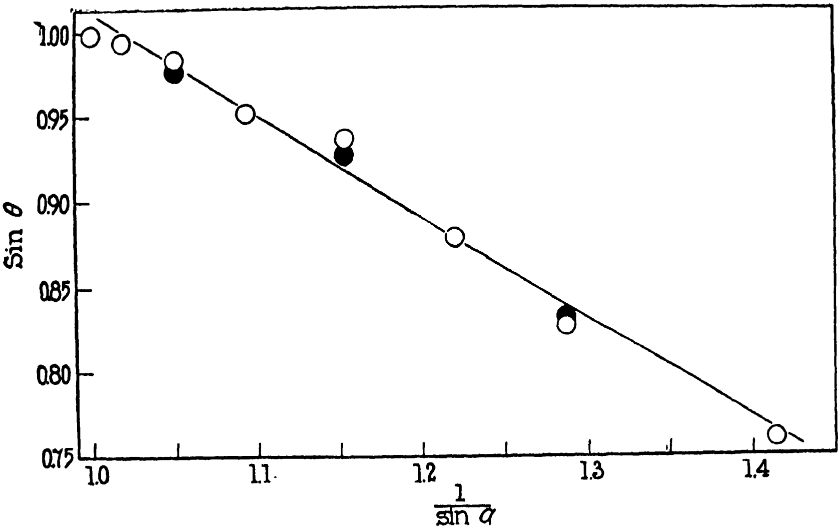

FIG. 5. The sine of the angle of orientation (θ) decreases in direct proportion to the reciprocal of the sine of the angle of inclination of the creeping plane.

Against this explanation it might be said that perhaps the cause for taking a certain angle (θ) at orientation is not dependent upon differences of pull on the two sides, or stress, or the head angle, but upon the pull working on the eyestalks. Several tests, however, with animals where the eyestalks were removed showed that they have no influence on the angle of orientation; the slugs describe, if they creep at all, the same angle (θ) as when the eyestalks are present.

SUMMARY.

On an inclined glass plate the slug *Agriolimax* orients and creeps upward or downward. The angle of orientation on the plane (Θ) is proportional to the logarithm of the component of gravity in the creeping plane. The coefficient of variability of the measured values of (Θ) decreases linearly as the logarithm at the gravity component in the creeping plane increases. The cosine of the angle of orientation decreases almost directly in proportion to the sine of the angle of inclination of the creeping plane to the horizontal, as previously found for young rats (Crozier and Pincus). But a more satisfactory formulation for the present case shows that the sine of the angle of orientation (Θ) decreases in direct proportion to the increase of the reciprocal of the sine of the angle of inclination of the creeping plane. This formulation is derived from the theory that the geotropic orientation is limited by the threshold difference between the pull of the body mass on the mutually inclined longitudinal muscles at the anterior end of the slug.

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THE OXYGEN CONSUMPTION OF FROG NERVE DURING STIMULATION.

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For many years the measurement of the possible increase in the oxygen consumption of nerves during activity has been a problem of serious difficulty. During last summer at Woods Hole I was able to overcome this difficulty by making use of the large nerves of the dogfish (following in this the lead of Parker (1925, *a*)) and by at the same time modifying Thunberg's micro respirometer so as to increase its sensitivity. In this preliminary work with the dogfish (Fenn, 1927) it was possible to demonstrate a sharp increase in the oxygen consumption at the beginning of stimulation, persisting for some time after the close of stimulation, but returning to the original rate within about 30 minutes or less. The excess oxygen taken in during activity was found on the average to be equal to 0.21 c.mm. per gm. of nerve per minute of stimulation, the resting rate being 1.35 c.mm. per gm. per minute. The absolute value of this excess oxygen is about 3 or 4 times too small to account for the heat production of nerve during stimulation as found by Downing, Gerard, and Hill (1926) in the frog nerve.

For purposes of comparison with these heat values it was important to obtain similar measurement of oxygen consumption on the frog nerve. These nerves being smaller, there was less likelihood of the oxygen tension being reduced to zero in the interior of the nerve trunk and hence a possibility of obtaining a greater excess oxygen consumption during stimulation. To improve matters still further I have studied the frog nerves in an atmosphere of oxygen, which was not easily available to me at Woods Hole. In spite of these modifications the figures now available for frog nerve are little larger than those obtained with the dogfish nerve, and the discrepancy between the values for oxygen and that for heat remains. At the present time the most obvious difference to which this discrepancy might be ascribed is the much greater duration of stimulation (20 to 30 minutes) necessary for determinations of oxygen as compared to the 10 seconds stimulation which suffices for heat measurements.

Method.

The method which I have used for frog nerve is the same as that previously described for dogfish nerve, with few modifications. The apparatus consists essentially of two 12 cc. bottles connected by a very fine capillary which carries a kerosene index drop. The nerve is laid on the stopper of one of the bottles in contact with sealed-in platinum electrodes. The other bottle serves for temperature compensation. Even so, a thermostat constant to less than 0.01°C. is necessary for accurate work, because chance currents in the bath affect the bottles independently. The temperature used was 22°C. throughout. Sodium hydroxide (M/4) is placed in each bottle to absorb carbon dioxide, so that the index drop, in response to the consumption of oxygen, will move toward the bottle containing the nerve. Positions of the drop are read at frequent intervals by means of a hand lens and two scales. One scale is just under the capillary the other 3 inches above it. In making a reading corresponding points on these two scales are kept in line, thus avoiding parallax. The capillary itself, with the lower scale, is under water. Further details of procedure may be found in the previous report.

The one difficulty encountered in using frog nerves in place of dogfish nerves is their small size. On the average the dogfish nerves weighed 5 times as much as those to be found in a good sized frog (30–40 mg. each). With the same apparatus, therefore, it should be possible to make corresponding measurements on four frog sciatic nerves and this turns out to be the case (Fig. 1, *a*). To avoid the labor of dissecting so many nerves it seemed worth while to try to make the apparatus still more sensitive. The instrument used for most of the measurements to be reported in this paper does not differ in general plan from the one previously described but it is smaller throughout. The bottles hold only 3.7 cc. instead of 12 cc. and the capillary (a piece of thermometer tubing) holds only 0.73 c.mm.

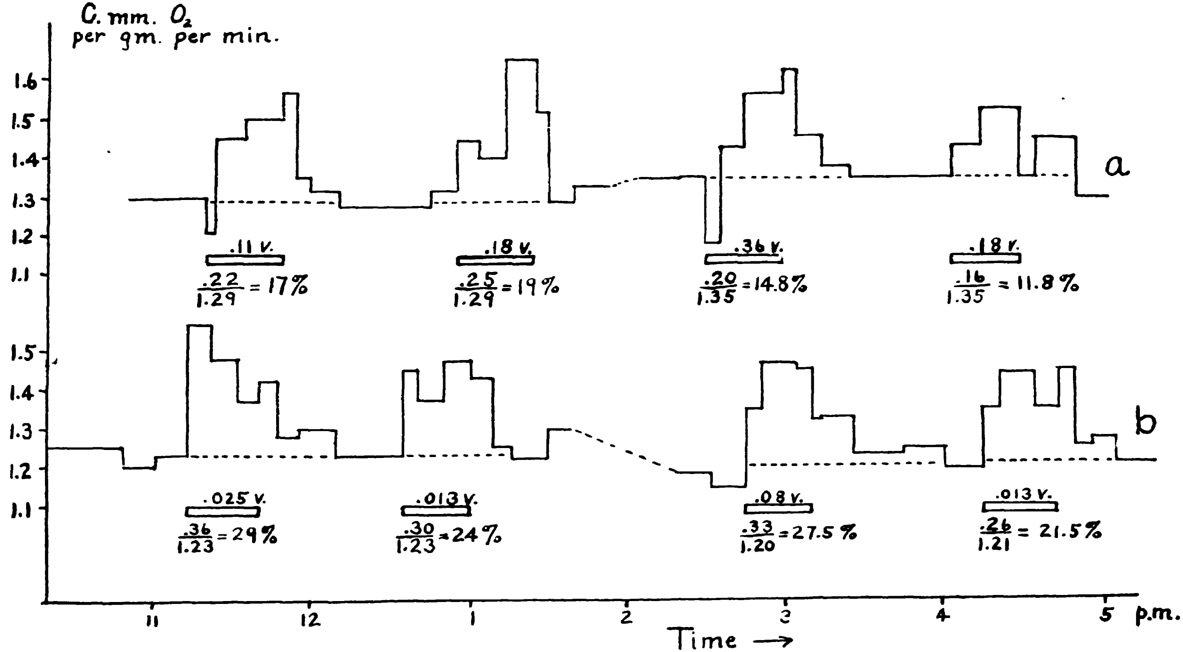

FIG. 1. Graphs showing the rate of oxygen consumption of frog nerves at rest and during stimulation periods of varying intensity. On the graph are inserted figures showing the equivalent voltage of the stimulating current derived from the secondary of an induction coil, and the percentage increase in the oxygen consumption for each period of stimulation. The total excess oxygen for each period of stimulation is represented by the area of the rise as plotted and seems to be independent of the intensity of stimulation within considerable limits.

per cm. instead of 1.94 as before. With this apparatus the index drop moves about 0.25 mm. (an amount easily measurable) per minute with a single sciatic nerve of 40 mg. in the bottle This threefold increase in sensitivity is not obtained without an increase in technical difficulties relating to the tiny index drop. A drop 2 mm. long is preferred. A longer one moves with too much friction in the small tube and a smaller one is harder to manipulate. Fortunately once a good drop is obtained it can be used indefinitely, barring accidents.

After dissection is completed the nerves are quickly weighed on a torsion balance and inserted in the apparatus so that only the central ends lie across the electrodes, the remainder of the nerve resting on the glass. The amount of solution in the two bottles is so adjusted that, after making allowance for the volume of the nerves, the air spaces are equal. Oxygen is then bubbled into the apparatus through the side arm and allowed to escape around the stopper. At the close of the experiment the nerve is weighed again. During the experiment a certain amount of blood and lymph drains out of the nerve so that the second weighing is 11 to 22 per cent (av., 16 per cent) lower than the original one. The final weight was always used in calculating the oxygen consumption. There was no perceptible drying out of the nerve in the apparatus.

Results.

The general character of the experiment does not differ from those previously reported on the dogfish nerve, except in regard to the heating effect of the stimulating current which was quite evident in the dogfish nerve but not usually perceptible in the frog nerve. The difference is partly due to the fact that a weaker stimulus was used for the frog (a Harvard induction coil set at 12 or 13 cm. (or tilted at an angle) instead of at 10 or 11 cm.), but mostly due to the larger size and hence smaller electrical resistance of the dogfish nerves. Moreover, the dogfish nerves were so long that they were brought into contact with the electrodes at more than one point, thus still further increasing the amount of current that could flow.

Intensity of Stimulation.—The results of two experiments with stimulation of varying intensity have been plotted in Fig. 1, *a* and *b*.

For *a*, four frog nerves were used in the dogfish-nerve apparatus. The stimulation periods are indicated by rectangular blocks and lasted usually 30 minutes. The figure over these blocks is the estimated equivalent voltage[1] of the stimulating current from the secondary of a Harvard induction coil vibrating at 50 per second and operated by a single dry cell. The figures underneath each stimulation block show the amount of increased oxygen measured in c.mm. per gm. of nerve per minute of stimulation, divided by the resting rate of oxygen consumption in c.mm. per gm. per minute, the quotient giving the percentage increase. No significant difference is to be seen between stimulation at 9 cm. coil distance (0.36 eq. volts) and at 10 or 11 cm., (0.18 and 0.11 eq. volts respectively), the figures for increased oxygen in these cases being 0.22, 0.25, and 0.20 c.mm. per gm. per minute of stimulation respectively. At the close of the experiment, 6 to 7 hours after dissection, this had dropped to 0.16, indicating perhaps a loss of function in some of the fibers.

In the experiment recorded in Fig. 1, *b*, two frog nerves were observed in the more sensitive apparatus. The nerves were stimulated with an induction coil as before, but a 100 per second tuning fork was used as an interrupter, there being about 0.5 volts across the primary coil during contact. The intensity of stimulation was varied by moving the secondary as before. No significant difference was found between stimulation with 11 cm. coil distance (0.08 eq. volts), 13 cm. coil distance (0.024 eq. volts), or 13 cm. coil distance with the coil tilted at 45° to the horizontal (0.013 eq. volts), the figures for these three cases being 0.36, 0.30, and 0.33 c.mm. per gm. per minute of stimulation. With an equivalent voltage of 0.013 volts the stimulus is too weak to be preceptible to the tongue, but nevertheless strong enough (at least the break shocks) to produce a maximal contraction of a frog sciatic-gastrocnemius preparation. Here again at the close of the experiment a somewhat smaller figure, 0.26 c.mm., was obtained with the weakest stimulation. The irregularities in these curves represent the experimental error of the method and are due to errors in reading the position of the drop and to small irregularities in its movement. The dotted line under each rise due to stimulation represents the base line which was used in calculating the magnitude of the increased oxygen usage. In the selection of this base line there is some uncertainty. The break in the graph in Fig. 1, *a*, shown by the dotted line, represents the time necessary to move the drop back to the other end of the capillary tube. This procedure frequently upsets the reading for a short time. A similar break in Fig. 1, *b*, is of similar significance but is longer because of some technical difficulties with the index drop. No systematic attempt has been made, beyond the experiments of Fig. 1, *a*, to measure accurately the effect of varying intensity of stimulation. The experiments here reported indicate that the differences, if they exist, are not large. This indeed is to be expected from the all-or-none law and is indirect evidence that the oxygen consumption increases here observed are actually due to nerve impulses and not to electrical or other artefacts at the electrodes.

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[1] The equivalent voltage corresponding to various settings of the secondary of the instrument used for these experiments has been determined by means of a thermal converter and a sensitive galvanometer. The equivalent voltage E is defined as the voltage necessary to force a direct current of the same strength through the secondary circuit. The P.D. across the primary terminals was 1 volt. The values of E for coil positions 13, 12, 11, 10, and 9 cm. were respectively 0.034, 0.055, 0.09, 0.15, and 0.3 volts. A heating effect due to high frequency radio waves direct from the spark gap without any electrical contact to the secondary has been allowed for.

It is quite certain that curves like those of Fig. 1 cannot be obtained from bits of cotton soaked in bicarbonate solution and laid across the electrodes, nor are they obtained from dead nerves. In one experiment, for example, the negative variation and the oxygen used were being recorded simultaneously. Stimulation produced no change in either. Because of a misunderstanding the silver electrodes used for recording the negative variation had not been washed free from the strong salt solution after plating; this oversight killed the nerve and unexpectedly afforded a clean-cut control experiment.

Frequency of Stimulation.

By stimulating the nerves at varying frequencies some data have been obtained which afford a fairly satisfactory proof that the extra oxygen is actually related to the energy requirements of the nerve impulse. For this purpose tuning forks vibrating at 100 and at

50 per second were used to interrupt the primary current. Thus 200 and 100 shocks per second were delivered to the nerve through the secondary coil. In Fig. 2 there are plotted two frequency curves to show the distribution of the results obtained with 32 stimulation periods at 100 interruptions per second and 21 periods with 50 interruptions per second. Comparing the averages of these results it is evident that doubling the number of impulses per second does not double the amount of extra oxygen used but increases it only 0.315/0.268 or 1.18 times. A similar result was obtained in one experiment in which the responses of the same nerve to the two rates of stimulation were directly compared, the current through the primary coil being equal for both frequencies. The result of this experiment is plotted in Fig. 3, *a*. In the two comparisons there recorded, doubling the frequency increased the excess oxygen used only 0.23/0.20 = 1.15 and 0.28/0.25 = 1.12 times. For purposes of comparison with these figures, the magnitude of the negative variation in nerves similarly stimulated was recorded with a Leeds-Northrup high sensitivity ballistic galvanometer of 2300 ohms resistance. The deflections obtained on stimulating the nerve with 200 shocks per

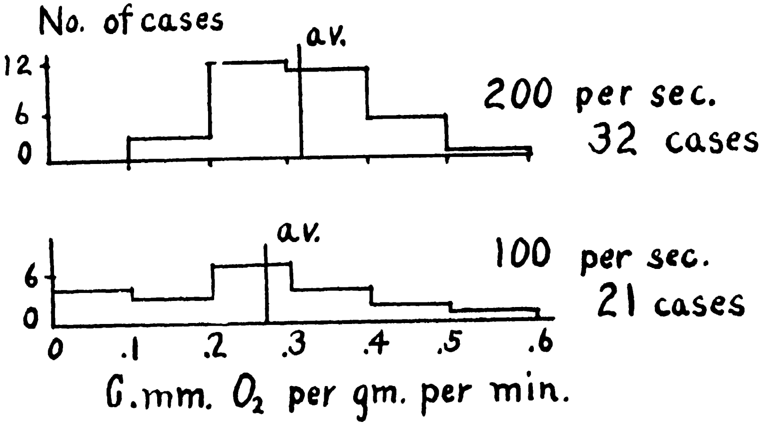

FIG. 2. Frequency diagrams to show the distribution of the values obtained for the excess oxygen consumption due to stimulation at 200 and at 100 shocks per second. The average values were respectively 0.315 and 0.268 c.mm. per gm. of nerve per minute of stimulation.

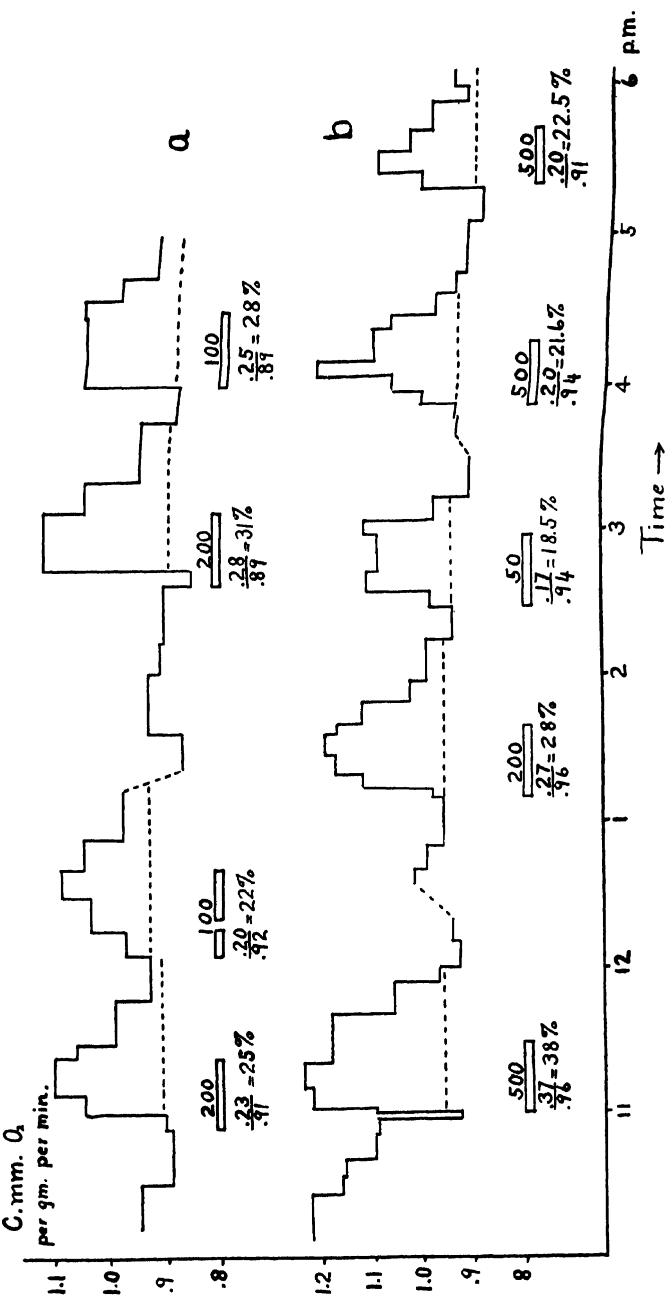

FIG. 3. Graphs showing the rate of oxygen consumption of frog nerves stimulated at different frequencies varying from 50 to 500 shocks per second. The intensity of stimulation was kept constant in each experiment, with the coil set at 13 cm. The voltage drop in the primary was 0.4 volts in *a* and 0.7 in *b*. The equivalent voltage varies in direct proportion to the frequency but for 200 per second it was 0.02 volts for *a* and 0.034 volts for *b*. Figures on the graph show the frequency of stimulation (above) and the percentage increase (below) calculated from the ratio of the excess oxygen per gm. per minute of stimulation and the resting rate per gm. per minute. In *b*, one end of the nerve was crushed which accounts, perhaps, for the high initial rate and led to a great uncertainty in the base line of the first period of stimulation.

second were on the average 1.15 times that found at 100 per second. Through the courtesy of Professor A. V. Hill I am informed that the heat production of nerve is increased 93/67 = 1.4 times for an increase in the frequency of stimulation from 100 to 280 per second or perhaps 1.25 times for an increase in frequency from 100 to 200. The fact that the response of the nerve to this change in frequency, as indicated by its oxygen consumption, is similar to its response as indicated by its negative variation and its heat production, is good evidence that the extra oxygen is actually used to supply energy for the nerve impulse.

In a few preliminary experiments simultaneous measurements have been made of the excess oxygen and of the negative variation on the same nerve. For this purpose two silver electrodes were introduced into the nerve chamber, one of which was in contact with the injured end of the nerve and the other with its intact surface. The results of one such experiment are plotted in Fig. 3, *b*, and the figures are collected in Table I. The values for the excess oxygen cannot be determined with great precision, but there does seem to be a definite correlation between the excess oxygen and the negative variation over this range of frequencies from 50 to 500 per second. The fact that this tenfold increase in the frequency had so little effect on the oxygen consumption is to be expected from the fact that impulses set up early in the refractory period are subnormal. It may be suspected also that with certain settings of a tuning fork vibrating at 250 per second there would be some interference between the make shock and its rapidly succeeding break shock.

TABLE I.

Frequency	Excess oxygen	Negative variation
per sec.	*c. mm.*	*m.v.*
500	0.37	2.12
200	0.27	2.09
50	0.17	1.2
500	0.20	2.16
500	0.20	1.92

In Fig. 4 there are plotted two frequency curves to show the range of values obtained for the percentage increase in oxygen consumption from stimulation at 200 shocks per second and for the resting oxygen consumption. The rather wide distribution is perhaps due to the varying conditions of the frogs (*R. pipiens*). For the earlier experiments they were kept in an indoor tank of running water; for the later experiments they were kept in water in a cold room maintained just above the freezing point, and were killed and dissected immediately after removal. No certain differences were noted in the behavior of the nerves in these two cases, however.

My most reliable data were obtained by stimulation at 200 shocks per second, and these may be used for comparison with the heat production of nerve. In 32 periods of such stimulation the resting rate was 1.23 c.mm. of oxygen per gm. of nerve per minute and the excess oxygen used in activity was 0.32 c.mm. of oxygen per gm. of nerve per minute of stimulation, or 26 per cent of the resting rate. The corresponding figures for the dogfish nerve were much the same, *i.e.* 1.35 c.mm. per gm. per minute as a resting rate and an increase on stimulation (100 shocks per second) of 0.21 c.mm. per gm. per minute of stimulation, the percentage increase varying from 10 to 32 per cent. Parker (1925, *b*) has reported a carbon dioxide elimination in the resting frog nerve of 4.46 c.mm. per gm. per minute and an increase due to stimulation of 14 per cent or 0.62 c.mm. per gm. per

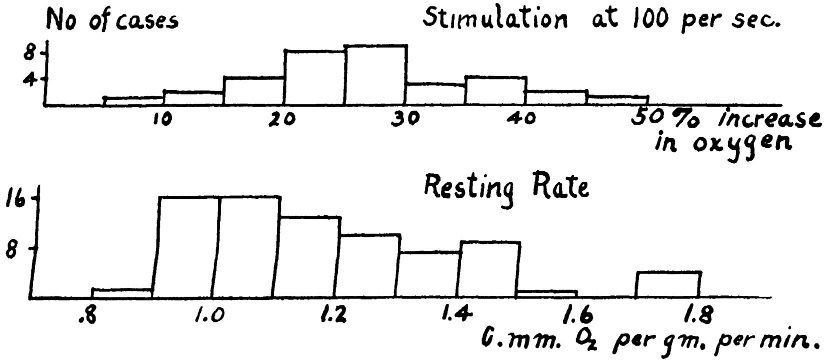

FIG. 4. Frequency diagram showing the distribution of the observed values for the percentage increase in oxygen consumption from stimulation at 200 per second (upper) and for the resting rate of oxygen consumption (lower).

minute of stimulation. In absolute magnitude these figures are higher than mine.

Downing, Gerard, and Hill (1926) found a heat production in the frog nerve which was equivalent to an oxygen consumption of 0.75 c.mm. per gm. nerve per minute of stimulation. They stimulated at a frequency of 280 shocks per second. At 100 per second the heat was 67/93 as great (personal communication from Professor Hill), which would have demanded an extra oxygen consumption of 0.54 c.mm. At 200 per second the figure would have been perhaps 0.66 c.mm. per gm. per minute of stimulation. This is about twice as large as the mean value which I have actually observed, *i.e.* 0.32 c.mm., although my highest figures have been over 0.5 c.mm. To account for this discrepancy, it is probable that during the first 10 seconds of stimulation there is a greater energy breakdown than during similar periods at the end of a half hour of stimulation. The heat was measured during the first 10 seconds only.

It is conceivable that even in the small frog nerve in an atmosphere of oxygen the central portion of the proximal end of the nerve, where its diameter is greatest, would be asphyxiated and fail to respond. This would help to explain the discrepancy between the heat production and the oxygen. By making use of Krogh's (1919) diffusion constant for oxygen, however, it can be shown that this is not the case. To do this one proceeds with a cylinder in much the same way that Warburg (1923) has done for the simpler case of a flat disc. Consider a cylinder of nerve of radius a and length l, consuming A cc. of oxygen per gm. per minute. D, the diffusion constant for oxygen in muscle tissues, $= 1.4 \times 10^{-5}$ cc. of oxygen diffusing across a surface area of 1 cm.² per minute under a pressure gradient of 1 atmosphere per cm. The concentration c_0 of oxygen at the surface is kept constant. Diffusion through the ends of the nerve is neglected. In any concentric cylindrical layer, of radii r and r-dr, the oxygen consumption in time dt is

$$A\,[\pi r^2 - \pi\,(r - dr)^2]\,l dt = 2\pi\,A\,lr\,dr\,dt \tag{1}$$

The oxygen diffusing *into* this layer in time dt is

$$D\,2\pi\,rl\,\frac{dc}{dr}\,dt \tag{2}$$

The oxygen diffusing *out* of this layer is

$$2\pi\, Dl\ (r - dr)\ dt\left(\frac{dc}{dr} - \frac{d^2c}{dr^2}dr\right) \tag{3}$$

Equation (1) = equation (2) − equation (3) or, after simplification,

$$\frac{Dd^2c}{dr^2} + \frac{Ddc}{dr} = Ar \tag{4}$$

The solution of this differential equation is

$$c = c_0 - \frac{A}{4D}(a^2 - r^2), \tag{5}$$

c being the concentration of oxygen in atmospheres at a distance r from the center of the cylinder, c_0 being the concentration at the surface, *i.e.* when $a = r$. Putting $r = 0$ and $a = 0.1$ cm. which is the maximum for frog nerves I have used,

$$c = 1 - \frac{0.00123 \times 0.1^2}{4 \times 1.4 \times 10^{-5}} = 1 - 0.22 = 0.78$$

atmosphere at the center of the nerve. Thus the tension inside the larger end of the nerve is 0.78 × 760 = 590 mm. if the nerve is in pure oxygen. If it is in air the tension at the center of the larger end is just reduced to zero.

From (5) it is evident that when in pure O_2 the tension at the center will just reach zero if

$$\frac{A}{4D}a^2 = 1 \text{ or if } a = 0.213 \text{ cm.}$$

which is about the maximum radius of the largest dogfish nerves. The assumption is made that A is independent of the tension of oxygen. This confirms the estimate previously made (Fenn, 1927) that the rate of diffusion of oxygen was a limiting factor in dogfish nerves in air.

I am indebted to an anonymous mathematical colleague for assistance with this equation.

SUMMARY.

1. The resting rate of oxygen consumption of the excised sciatic nerve of the frog is 1.23 c.mm. of oxygen per gm. of nerve per minute.

2. During stimulation with an induction coil with 100 make and 100 break shocks per second there is an excess oxygen consumption amounting on the average to 0.32 c.mm. of oxygen per gm. of nerve per minute of stimulation, or a 26 per cent increase over the resting rate.

3. The magnitude of the excess oxygen consumption in stimulation, in agreement with the all-or-none law, is not markedly influenced by considerable variations in the intensity of stimulation.

4. Increasing the frequency of stimulation from 100 to 200 shocks per second increases the extra oxygen used only 1.12–1.18 times. The same change in frequency of stimulation increases the negative variation 1.15 times and the heat production about 1.25 times (Hill).

5. This parallelism between the excess oxygen and the negative variation argues definitely for some causal connection between the excess oxygen and the nerve impulse itself.

6. Calculation shows that the oxygen tension inside these nerves was not zero.

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THE KINETICS OF DARK ADAPTATION.

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I.

Nature of Contents.

1. Purpose.—Many photosensitive animals acquire in the dark an increased sensibility to light. A quantitative description of this process of dark adaptation is limited, however, to very few animals. For vertebrates, there are the numerous and accurate measurements with man (Piper, 1903; Nagel, 1911; Hecht, 1921–22); the few observations with the chick (Honigmann, 1921); and the measurements with the tadpole (Obreshkove, 1921); while for invertebrates there are only the data with the clam, *Mya arenaria* (Hecht, 1918–19 *b*). Additions to this meager collection of data are obviously desirable. The first purpose of the present paper is to present measurements of the dark adaptation of two additional invertebrates: the lamellibranch, *Pholas dactylus;* and the ascidian, *Ciona intestinalis.*

The original study of the dark adaptation of *Mya* (Hecht, 1918–19 *b*) contained an analysis of the process in terms of a photosensory mechanism. Since then, much has been learned about the nature of the processes involved, and as a result certain modifications in the details of the theoretical treatment have become necessary (Hecht, 1922–23). The second purpose of this paper is to present an analysis of the data of dark adaptation which is in keeping with present knowledge.

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† The experiments here recorded were made at the Zoological Station, Naples, during my tenure of the Jacques Loeb Memorial Table. I wish to express my appreciation of the many kindnesses shown me during my stay in Naples by the Director of the Zoological Station, Dr. Reinhard Dohrn.

The organisms whose dark adaptation has been measured may be divided into two groups depending on the nature of their photosensitive structure, and on the method of making the measurements. In man and the chick the light sensitive system is a complicated vertebrate eye. Dark adaptation in these animals has been measured by finding the illumination intensity just perceptible to the eye during different moments of the stay in the dark. Thus of the two factors which control the photochemical effect of a given light. the time of exposure is held constant while the intensity is varied However, in the other animals mentioned the photoreceptors are superficially located and are diffuse. In *Mya* and *Pholas* they are on the siphons and on the exposed parts of the mantle (Dubois, 1892; Wenrich, 1916); in *Ciona* they are in a small area between the siphons (Hecht, 1918–19 *a*); and in the tadpole they are in the skin (Obreshkove, 1921). With these animals dark adaptation is measured by finding the exposure required to elicit a response to a given illumination. Time is thus the variable while the intensity is constant.

The principles underlying the theoretical analysis of dark adaptation is the same in these two groups of animals (Hecht, 1919–20). But because of the difference in the method of securing the data for the two classes it is more convenient to present them separately. I shall therefore consider here only the second group. The third purpose of the present paper is to complete the analysis of this class of animals by including a study of Obreshkove's data of the dark adaptation of the tadpole.

It might appear desirable, in order to render this study complete, to collect here the bits of information about dark adaptation which are scattered in the voluminous literature of the sensitivity of animals to light. However, such a course would not be profitable, because this material consists mostly of the mere statement that a given animal can become dark adapted, coupled only occasionally with a numerical datum giving the order of magnitude of this effect (*e.g.* Hess, 1910). An apparent exception might seem to be the work of Folger (1924–25) who, under the heading of dark adaptation records a few measurements of the recovery of *Ameba* from stimulation by light. Folger has very clearly shown, however, that most of this recovery can occur in the light as well as in the dark. The process is, therefore, not dark adaptation; and is not relevant to the present study dealing with the quantitative aspects of this process.

2. *Definitions.*—Folger's paper calls for comment here, because of the unexpected meaning which he has attached to the terms dark adaptation and light adaptation.

> "An organism is said to be dark adapted when it will respond to a sudden increase in the intensity of illumination. The converse of dark adaptation is light adaptation. This is brought about by exposure to light. An animal is said to be light adapted when from a lack of dark adaptation it fails to respond to a sudden increase in illumination."[1]

According to Folger's definition of dark adaptation, the human eye, or *Mya*, or *Ciona* are dark adapted in broad daylight, since they all respond to a sudden increase in illumination under such conditions. Similarly according to Folger, none of these organisms can become light adapted, since there are no light conditions known under which they do not respond to a sudden increase in illumination if of sufficient magnitude. It is therefore to be regretted that an erroneous and confused connotation has been applied to such terms as dark and light adaptation which have always had a precise and accepted meaning.[2]

Aubert (1865) introduced the term adaptation into physiology when he recorded the first measurements of the dark adaptation of the eye. Since then there has been no misunderstanding of its meaning. A detailed treatment of it has been given by Nagel (1911). This is too long to be quoted here; I shall therefore summarize it briefly in a form applicable to the present situation. A dark adapted animal is one whose sensitivity to light has reached a constant value as the result of a sustained stay in the dark. A light adapted animal is one whose sensitivity to light has reached a constant value as the result of a sustained exposure to a given intensity of illumination. The process of reaching these two states is that of dark adaptation and of light adaptation, respectively. These definitions might be supposed to be self-evident. The terms which they describe will therefore be used in their accepted sense in the present paper.

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[1] Folger (1924–25, p. 279).

[2] This confusion is only one of the many to be found in Folger's paper,—a situation which may be best illustrated perhaps by quoting one of the major conclusions of the work (p. 290). Folger is concerned with the fact that "*A mechanical shock exerts a distinct effect upon the reaction to light.*" "*This indicates that increase in illumination and mechanical shock produce the same changes in Amoeba, that mechanical stimulation and photic stimulation are fundamentally the same. If this is true, it is evident, since mechanical stimulation is not photochemical, that photic stimulation cannot be photochemical.*"

II.

Experiments with Pholas dactylus.

1. General Properties of Sensibility.—Pholas is an animal whose photosensory behavior is similar to that of *Ciona* and *Mya*. Its resemblance to *Mya* in particular is so extraordinary that in a short time it is possible to demonstrate in a qualitative way the existence of all the interrelations which have been quantitatively established in *Mya* (Hecht, 1925). The following are some of these properties; they are recorded here so that the study of the dark adaptation of *Pholas* may be intelligible.

(*a*) *Pholas* is very sensitive to light, and responds to it by a vigorous retraction of its extended siphon. The time from the beginning of the exposure to the beginning of the retraction was called the "latent time" by Dubois (1892) who first worked with this animal. In conformity with the less accurate but more usual practice it will be referred to as the "reaction time."

(*b*) This reaction time is not a simple period. As in the case of *Ciona* and *Mya*, it is composed of two parts, an exposure period and a latent period. The first is occupied by the necessary exposure to light. It varies with the intensity and can be very short with high intensities. The second, or latent period, occupies most of the reaction time. During the latent period the animal may remain in the dark and still respond after the usual reaction time. Thus a reaction time of 2 seconds to an illumination of 500 meter candles is composed almost entirely of latent period, because the actual exposure to light need be only 0.02 second.

(*c*) Up to a certain limit of exposure the latent period varies inversely with the duration of the exposure.

(*d*) Temperature has almost no influence on the exposure period, as is to be expected if the exposure is concerned with a photochemical reaction. The latent period, however, is definitely influenced by temperature, in a manner similar to most "dark" reactions.

(*e*) *Pholas* comes into sensory equilibrium with any illumination to which it is exposed continuously. Its first act on being illuminated is to retract its siphon. In a few seconds, however, it extends the siphon and appears to be unstimulated by the light. Its sensitivity is now much less than before, because the intensity of illumination has to be considerably augmented in order to cause *Pholas* to respond again.

(*f*) Its original sensibility may be restored by placing the animal in the dark. This is a fairly slow process in *Pholas*, as will be apparent presently.

Dubois (1892) failed to note many of these properties of the sensory process, because of his interest in the contraction of the siphon. He laid great stress on the fact that the siphon response may be recorded graphically, and most of his work was concerned with the form of the siphon contraction under different conditions of stimulation. To Dubois is due the important point that the photosensory process and the resulting retraction response are essentially independent of the rest of the animal, because he showed that a detached siphon retains its sensibility to light and its capacity to contract for several days after removal from the rest of the animal.

2. *Dark Adaptation.*—If after exposure to strong light, *Pholas* is placed in the dark, its reaction time to a light of constant intensity decreases steadily until it reaches a constant minimum characteristic of its response to that intensity. The course of this dark adaptation has been investigated in the following manner. An animal is exposed to an illumination of 10,000 meter candles. After the response, it almost at once expands its siphon. 2 or 3 minutes seem to be sufficient for light adaptation, but 7 minutes are allowed in these experiments. The animal is then placed in the dark and during the next 2 hours its reaction time to an illumination of 30 meter candles is measured three times. Several hours later the animal is again light adapted as before; it is placed in the dark and its reaction time to 30 meter candles is measured at such times that all together there are secured six values of the reaction time at half hour intervals during dark adaptation. The measurements are made in two series instead of one, so as to

allow an hour between successive exposures. In this way the progress of dark adaptation is disturbed as little as possible by the short exposures to light needed for making the measurement.

During the stay in the dark the animal in its rectangular glass dish is kept in a water bath so as to maintain the temperature constant. The source of light is properly screened in a box, and water filters are interposed to reduce the heat to a minimum. Though negligible during a measurement, this factor must be controlled during the 7 minutes light adaptation when the temperature is kept constant by the addition of cold sea water to the dish.

TABLE I.

Dark Adaptation of Pholas. 18 Animals. Temperature, 16.5°C. Latent Period, 1.00 Second. $k = 0.0143$; $a = 0.730$.

Time in dark t	Reaction time r	
	Observed	$r = \frac{95.8 + t}{25.9 + t}$
min.	*sec.*	*sec.*
10	2.95	2.95
30	2.23	2.25
60	1.81	1.81
90	1.65	1.60
120	1.46	1.48
150	1.40	1.40

With *Pholas* I measured the dark adaptation of 18 animals. The data secured are given in Table I, and graphically in Fig. 1. It is apparent that the process is regular, and at this temperature takes more than 3 hours to become complete.

3. Theoretical Analysis of Dark Adaptation.—The reaction time of *Pholas* to light is an interval occupied by several processes which combine to produce a constant effect, namely a siphon retraction. The algebraic sum of the velocities of these separate processes is represented by the reciprocal of the reaction time. Only two of these processes, the photochemical and the latent period, need be considered, since the time occupied by conduction and central nervous activity is negligible in stop-watch measurements and may be included in the latent period. The data therefore show that the sum of the velocities of the two major processes increases during dark adaptation.

The work with *Mya* (Hecht, 1922–23; 1923–24) has shown that to cause a given photosensory response a definite and constant amount of photochemical decomposition must be produced by the incident light. Let us assume that this holds during the dark adaptation of *Pholas*, and therefore that the photochemical effect during each reaction time in Table I is constant. This is the only assumption which has to be made in order to understand dark adaptation.

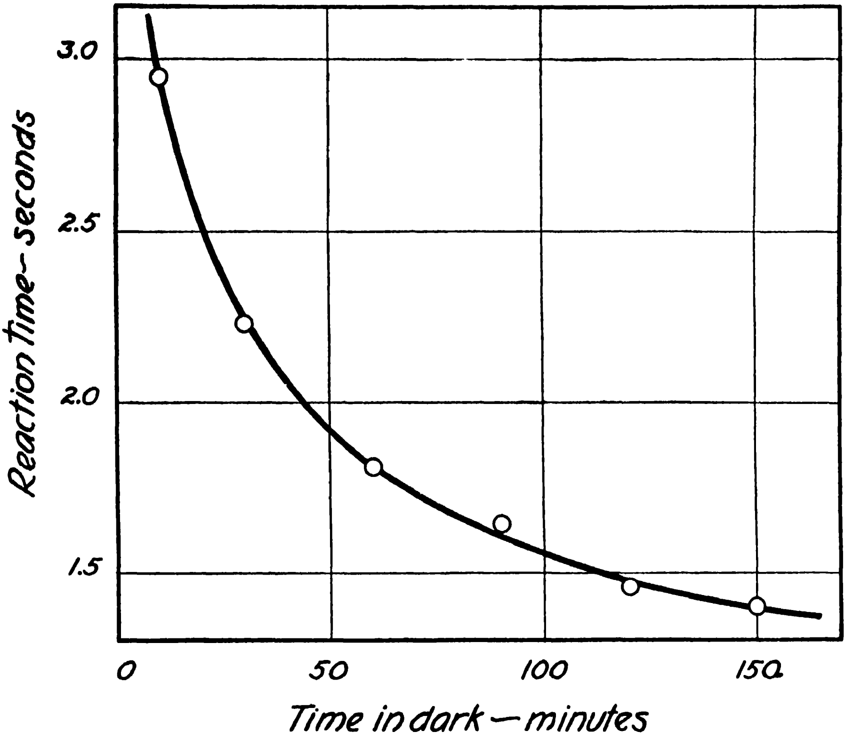

FIG. 1. Dark adaptation of *Pholas*. Each point is the average of 18 measurements, one with each of 18 animals. The first, third, and fifth points on the curve for each animal were made during one run of dark adaptation, while the other three points were made during another run several hours later. The curve is calculated in terms of a bimolecular reaction.

It is already well known that the velocity of the latent period is directly proportional to the magnitude of photochemical effect produced during the exposure (Hecht, 1918–19 *c*; 1925–27). If the photochemical effect be assumed constant, it follows that the velocity of the latent period is also constant.[3] The total velocity of the reaction time processes is thus composed of two velocities, one of which is constant. Any increase in total velocity must therefore represent an increase in the velocity of the primary photochemical process only; and hence the curve in Fig. 1 represents the changes in the velocity of the photochemical reaction during dark adaptation.

Since the intensity of the measuring light is constant, the change in velocity of the primary photochemical reaction is very likely due to a change in the concentration of sensitive substances accumulating during dark adaptation. On obvious photochemical grounds, it may be supposed that the velocity of the reaction is proportional to the concentration of photosensitive substance in the sense cells.[4] It then follows that the concentration of sensitive material increases in conformity with the shape of the experimental curve of dark adaptation. When the form of the curve in Fig. 1 is investigated it is found to be that of a bimolecular reaction isotherm

$$k_1 = \frac{x}{t\,a\,(a - x)} \tag{1}$$

where k is the velocity constant; t the time of dark adaptation; x the concentration of photosensitive material already formed; and $a - x$

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[3] This does not mean that the duration of the latent period is necessarily constant during the large changes in reaction time associated with dark adaptation. In fact, it is known that the latent period is not quite constant under such conditions (Hecht, 1922–23, p. 573). What is constant is the average velocity of the latent period process. It has been shown experimentally (Hecht, 1918–19 *c;* 1925–27) that the reaction underlying the latent period depends for its progress on the products formed by the photochemical reaction during the exposure. If the exposure is long, the latent period reaction begins as soon as some photochemical products are formed, and increases in velocity as these increase in concentration. The average velocity of the latent period process is then constant, though its actual duration may vary slightly. If, however, the necessary exposure is short in comparison with the latent period the former may be considered instantaneous, and therefore both the velocity of the latent period and its duration will be constant.

[4] This proportionality between concentration of sensitive material and velocity of reaction is a first approximation, and holds strictly in an irreversible reaction only. However, since the analysis in terms of it is descriptive of the data, it is retained in its simple form without the added term for the reversible reaction.

the concentration still to be formed. The third column in Table I gives the calculated values of the reaction time on which the theoretical curve in Fig. 1 is based.

It is necessary to describe in detail the method of calculating the terms in the above equation in order to show how they are derived from the experimental data of Table I. The limits between which the velocity of the reaction time processes can vary are the minimum velocity at the first moment of dark adaptation, and the maximum possible velocity as given by the velocity of the latent period alone. The range between these two limits represents the possible variation in the speed of the photochemical reaction, and corresponds to the total change a in concentration of sensitive material S. If x is the concentration of S at the moment t, then $a - x$ is the concentration of S still to be formed, and corresponds to the difference between the maximum velocity and the velocity at the moment t; *i.e.*, the difference between the reciprocal of the latent period and the reciprocal of the reaction time at the moment t.

TABLE II.

Determination of Latent Period of Pholas. Temperature, 16.5°C. 16 Animals.

Exposure	Reaction time	Latent period
sec.	*sec.*	*sec.*
0.11	1.47	1.36
0.20	1.37	1.17
0.28	1.28	1.00
0.35	1.27	(1.00)
0.41	1.28	(1.00)

The reaction time values are of course the measurements in Table I. The latent period is determined by a separate experiment with the same animals. An animal is stimulated at hourly intervals by a series of graded exposures, and the reaction time measured. Table II gives the data for 16 of the animals used in Table I. It is apparent that the smallest exposure giving the minimum reaction time of 1.28 seconds is 0.28 second. The minimum reaction time minus the minimum exposure is obviously the latent period, and is equal to 1.00 second.

The value $a - x$ at the moment t is thus a difference between the reciprocals of two experimentally determined magnitudes. If now $\frac{1}{a - x}$ is plotted against t the points should lie on a straight line

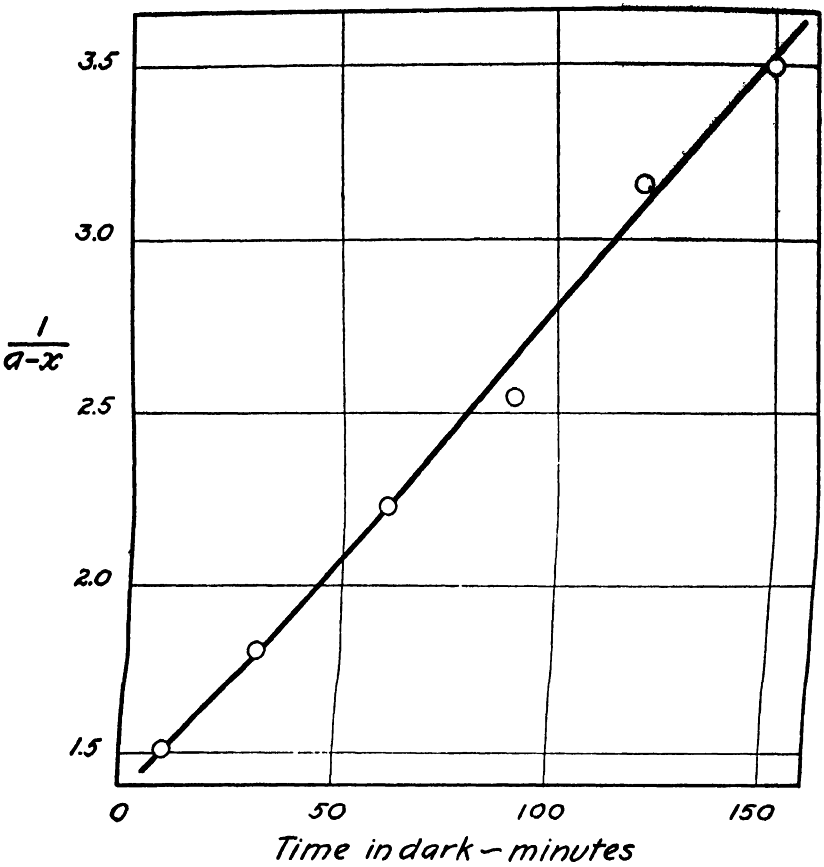


FIG. 2. Dark adaptation of *Pholas*. Same data as in Fig. 1. The ordinates are $rp / (r - p)$, which is equivalent to $1 / (a - x)$ in the equation for a bimolecular process. Here r is the reaction time and p the latent period.

provided the reaction is bimolecular. This is because equation (1) can be converted into the form of a straight line

$$\frac{1}{a - x} = kt + \frac{1}{a}. \tag{2}$$

If we call r the duration of the reaction time, and p that of the latent period, then $a - x$ will be represented by $1/p - 1/r$, and $\frac{1}{a - x}$ by $\frac{rp}{r - p}$ according to the above reasoning. Fig. 2 shows that if $\frac{1}{a - x}$ or its equivalent $\frac{rp}{r - p}$ is plotted in this way, the data conform to equation (2). The tangent of the line is k, the reciprocal of its intercept on the ordinate axis is a. The significance of this graphic mode of computation is that the values of k and a are both found directly from the experimental data, and are not arbitrarily assumed in the calculations.

4. *Interpretation.*—In making the above analysis no assumption was made with regard to the way in which the sensitive material accumulates in the sense cells during dark adaptation. The agreement between the kinetics of a simple bimolecular reaction and the course of dark adaptation permits one to draw the conclusion that the process is chemical in nature. If the kinetics of dark adaptation had followed a monomolecular isotherm this interpretation would have been doubtful because under certain conditions the course of a diffusion process may also be described that way. As it is one must suppose that two substances at least are concerned in the formation of the sensitive material, and that these two substances combine in a chemical manner.

It is apparent that the chemical nature of the process can be corroborated by studying the effect of temperature on dark adaptation. This was not done with *Pholas*, but has been done with *Mya*, and the results as given in the next section bear out the chemical interpretation of the present data.

III.

Mya arenaria.

1. *Assumptions.*—The original study with *Mya* contained an analysis of its dark adaptation on the basis of two assumptions. These were first, that the photochemical effect of light on the sensory system is directly proportional to the exposure; and second, that the concentration of photochemical decomposition products required for a

response is proportional to the concentration of products already present in the sense cell. In terms of them dark adaptation was shown to follow the kinetics of a bimolecular reaction.

However, both assumptions have proven inadequate for the further handling of the characteristics of the photosensory process, and have been discarded (Hecht, 1922–23). Therefore, it is proposed to show here how from the same data the same conclusion may be reached by the simpler and more plausible analysis just used with *Pholas*. This entails only one assumption: under all circumstances a definite amount of photochemical decomposition is required to produce a given sensory effect. Though given here as an assumption purely, its validity is attested by a good deal of experimental evidence (Hecht, 1922–23; 1923–24).

TABLE III.

Dark Adaptation of Mya. Data from Hecht. 6 Animals. Temperature, 22.0°C.*

Time in dark	Reaction time		
	Observed	Calculated in original way	Calculated in present way
min.	*sec.*	*sec.*	*sec.*
4	2.28	2.21	2.24
8	1.95	1.92	1.94
14	1.70	1.71	1.74
20	1.60	1.60	1.63
29	1.51	1.53	1.55
40	1.50	1.48	1.49
53	1.50	1.47	1.45

* *Hecht (1918–19 b), p. 551.*

2. *Dark Adaptation.*—The data, given in Table III, are the original ones for *Mya*, and were secured by methods which are essentially those described above for *Pholas*. With the data are the values calculated in the present manner as given in the previous section with *Pholas*, and in the old manner. It is clear that the two modes of theoretical treatment give the same approximation to the experimental data. This follows from the fact first, that the curve of dark adaptation is an hyperbola, and second, that the two methods of

calculation involve the use either of a given value of the ordinates (reaction time) or of its reciprocal.

It may not be amiss to indicate precisely why the two methods give almost identical results. Let r_t be the reaction time after t minutes of dark adaptation, and t_o its value at the first moment when $t = 0$. Let p be the latent period. Then according to the original hypothesis

$$a = k_2 (r_o - p) \tag{3}$$

$$x = k_2 (r_o - r_t) \tag{4}$$

$$a - x = k_2 (r_t - p) \tag{5}$$

where a is the total amount of sensitive material to be formed, x the amount already formed, and $a - x$ the amount still to be formed from the precursors according to the usual equation

$$k_1 = \frac{x}{t\,a\,(a - x)} \tag{1}$$

for a bimolecular process. If the corresponding values in (3), (4), and (5) are substitued in (1) it becomes

$$k = \frac{r_o - r_t}{t\,(r_o - p)\,(r_t - p)} \tag{6}$$

which describes the behavior of the experimental data. According to the present alternative hypothesis it is

$$a = k_3\left(\frac{1}{p} - \frac{1}{r_o}\right) \tag{7}$$

$$x = k_3\left(\frac{1}{r_t} - \frac{1}{r_o}\right) \tag{8}$$

$$a - x = k_3\left(\frac{1}{p} - \frac{1}{r_t}\right) \tag{9}$$

which describe the relations, and which give

$$k = \frac{\frac{1}{r_t} - \frac{1}{r_o}}{t\left(\frac{1}{p} - \frac{1}{r_o}\right)\left(\frac{1}{p} - \frac{1}{r_t}\right)} \tag{10}$$

when substituted in equation (1). Equation (10) reduces very [illegible] to

$$\frac{k}{p^2} = \frac{r_0 - r_t}{t\,(r_0 - p)\,(r_t - p)} \qquad (11)$$

which, since p is constant, is identical in form with equation (6). The choice between the two hypotheses is therefore not so much as to which gives a better agreement with the data of dark adaptation, but as to which is more consistent with other work, and inherently perhaps more plausible.

TABLE IV.

Dark Adaptation of Mya at Different Temperatures. Data from Hecht. 5 Animals.*

11.5°C.; k = 0.0744			16.2°C.; k = 0.124			21.9°C.; k = 0.217		
Time in dark	Reaction time		Time in dark	Reaction time		Time in dark	Reaction time	
	Observed	Calculated		Observed	Calculated		Observed	Calculated
min.	*sec.*	*sec.*	*min.*	*sec.*	*sec.*	*min.*	*sec.*	*sec.*
11.	5.04	4.72	4.	3.50	3.56	4.	2.30	2.25
15.	4.13	4.31	8.	2.98	2.97	8.	1.98	1.95
20.	3.94	3.94	13.5	2.44	2.56	13.	1.70	1.76
29.5	3.50	3.48	20.	2.30	2.31	19.5	1.64	1.64
39.5	3.11	3.20	30.	2.12	2.10	29.	1.54	1.55
55.	2.92	2.92	40.5	2.05	1.98	39.5	1.52	1.49
			54.	1.90	1.89	54.5	1.45	1.45

* Hecht (1918–19 *b*), p. 556.

3. Dark Adaptation and Temperature.—The present theoretical analysis of the data of *Mya* brings to light a quantitative relation between dark adaptation and temperature. The purpose of the original experiments with temperature was to measure the temperature coefficient of dark adaptation in order to ascertain whether the process underlying dark adaptation is chemical in nature, in this way corroborating the evidence from its kinetics. The temperature coefficient, Q_{10}, for the velocity constant k confirmed this by being near 2.5. Nevertheless the values of k as derived from the original computations did not fit the Arrhenius equation relating temperature with the velocity constant of a chemical reaction (Arrhenius, 1912).

It is a significant point that recalculation of the same data in terms of the present analysis yields a series of values for the velocity constant k which show an excellent agreement with the Arrhenius equation.

The experimental data for dark adaptation at three temperatures are given in Table IV. They are shown graphically in Fig. 3 according to the method of analysis described above. The corresponding calculated values are also given in Table IV, from which, as well as from Fig. 3, may be noted the adequacy of a bimolecular reaction for describing dark adaptation.

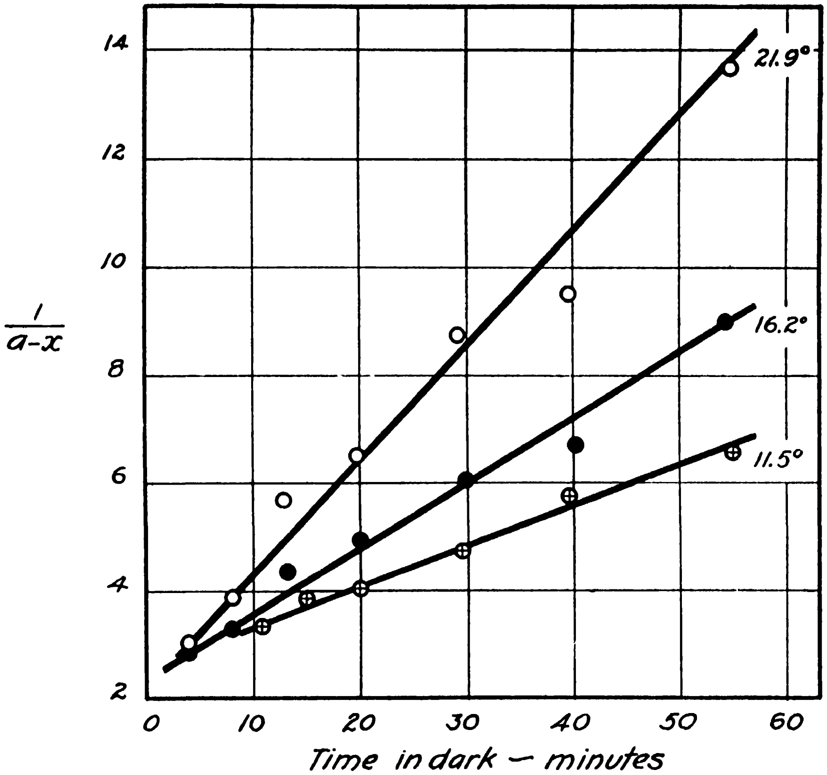


Fig. 3. Dark adaptation of *Mya* at three temperatures. The ordinates are $rp / (r - p)$ which is equivalent to $1 / (a - x)$ in the equation for a bimolecular process. The velocity of the reaction as indicated by the slope of the lines increases with the temperature.

The velocity constants k derived from these data are given in

Table IV, and their relation to the temperature in Fig. 4. For purposes of graphic presentation the Arrhenius equation may be written

$$\ln k = -\frac{\mu}{RT} + C \tag{12}$$

where k is the velocity constant at the absolute temperature T, C is a constant of no significance here, R is the gas constant, and μ is the critical increment or temperature characteristic (Crozier, 1924). A plot between $\ln k$ and $1/T$ should be a straight line whose tangent is μ. The data yield such a relation with μ = 17,400. In judging of the reliability of the three points through which the straight line passes, it is to be remembered that each velocity constant k is computed from at least 6 points on the dark adaptation curve, each point being the average of 5 measurements.

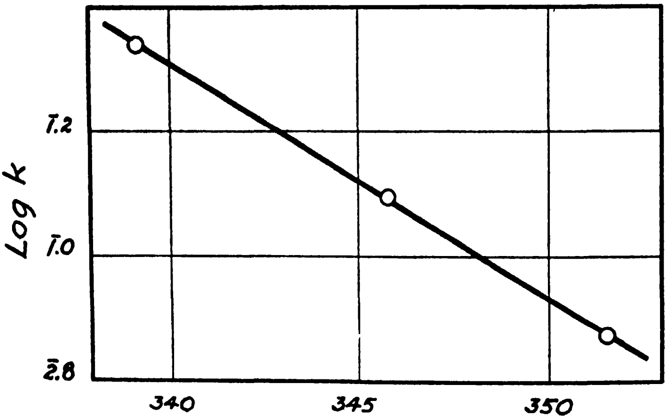


FIG. 4. Relation between temperature and the velocity constant k of the dark adaptation reaction. The straight line represents the Arrhenius equation.

The effect of temperature on dark adaptation in Mya, therefore, not only shows the process to be bimolecular at all temperatures investigated, but demonstrates as well that the velocity constant of the process bears a well known theoretical relation to the temperature such as is usually found in chemical reactions.

IV.

Ciona intestinalis.

1. Method.—In presenting the data obtained with *Pholas* and *Mya* only the barest manipulative essentials have been mentioned. Both species, though very sensitive to light, are hardy laboratory animals and tolerate much handling without change of sensibility. Therefore the methods used are simple and can easily be acquired anew by any one wishing to repeat the experiments.

The problem with *Ciona*, however, is more difficult. The species is not very sensitive to light, and in order to measure its dark adaptation a high illumination is required. This involves a still higher illumination for the initial light adaptation. The animals must therefore be brought close to a powerful source of light, such as a 1000 watt, gas-filled lamp of 2000 candle power. To maintain the animal at a constant temperature the usual heat screens are not enough, and a continuous addition of ice-cold sea water has to be maintained. On the other hand, *Ciona* is very sensitive to mechanical disturbances, and is easily stimulated by the necessary stirring of the water in the dish. Moreover, a sudden exposure to high illumination for light adaptation makes it contract vigorously; and in contrast to *Pholas* and *Mya* it does not relax immediately on continued illumination, and may remain contracted as long as 15 or 20 minutes. During this period its own water current is so small that stirring must be more continuous than usual, which serves to stimulate it mechanically and prolong the contracted state. Therefore to secure reproducible and significant data with *Ciona* it has been necessary to devise an especially careful mode of experimentation.

About 25 animals are isolated, each in a separate rectangular dish 5 cm. wide, 10 cm. long, and 7 cm. high, and kept in the dark overnight. Next morning their reaction time to a standard illumination is measured; as a result of which it is possible to select 5 or 6 animals which give a vigorous and clean-cut response to illumination, and which do so in about the same reaction time. Each animal so selected is then carried through the following steps.

It is light adapted. The intensity of the lamp is reduced by means of a rheostat until the filament barely glows. *Ciona* may be brought as close as 30 cm. to such a light without responding. During a period of half an hour the current is increased up to 200 volts so gradually that the animal does not at any time contract to light, and at the end of the period it is illuminated with 10,000 meter candles. The temperature is kept constant by the very careful addition of small amounts of ice-cold sea water which is distributed by the vigorous current of the fully expanded animal. The animal remains at this high illumination for 6 minutes. At a given moment the illumination is discontinued sharply by means of a shutter, and dark adaptation begins. The manipulations from now on are carried on by the light of a dim ruby lamp to which *Ciona* is not sensitive.

The animal in its dish is placed in complete darkness in a thermostat and kept at a constant temperature for the next 7 or 8 hours. At certain times during dark adaptation the dish is carefully taken out and placed near the lamp in such a position that the animal will receive on exposure an illumination of 6000 meter candles. A minute is allowed to elapse; the animal is exposed by means of a shutter; its reaction time is recorded with a stop-watch; the light is turned off immediately; and the animal is returned to the thermostat. The exact moments when these measurements are made vary somewhat in the different series, but are the same for all the animals in a series. The first measurement is usually made 15 or 20 minutes after the beginning of dark adaptation; the remaining ones at hourly intervals thereafter. If it is desired to have readings closer together the entire procedure of light and dark adaptation is repeated the next day, and readings are made at intervals between those taken the day before.

2. Data.—After many preliminary experiments involving the elimination of errors and development of technic, I measured the dark adaptation of 61 individuals grouped in ten series of experiments. To illustrate the type of result secured with *Ciona* the detailed data of one series of experiments are given in Fig. 5. It is to be noted about Fig. 5 that (*a*) the points given are individual measurements of the reaction time, not averages; (*b*) each animal was measured on 2 successive days, three readings being made each day; (*c*) the curve drawn through the points for each animal is a theoretical one derived as will presently be described. This series is typical; five or six similar ones could be given.

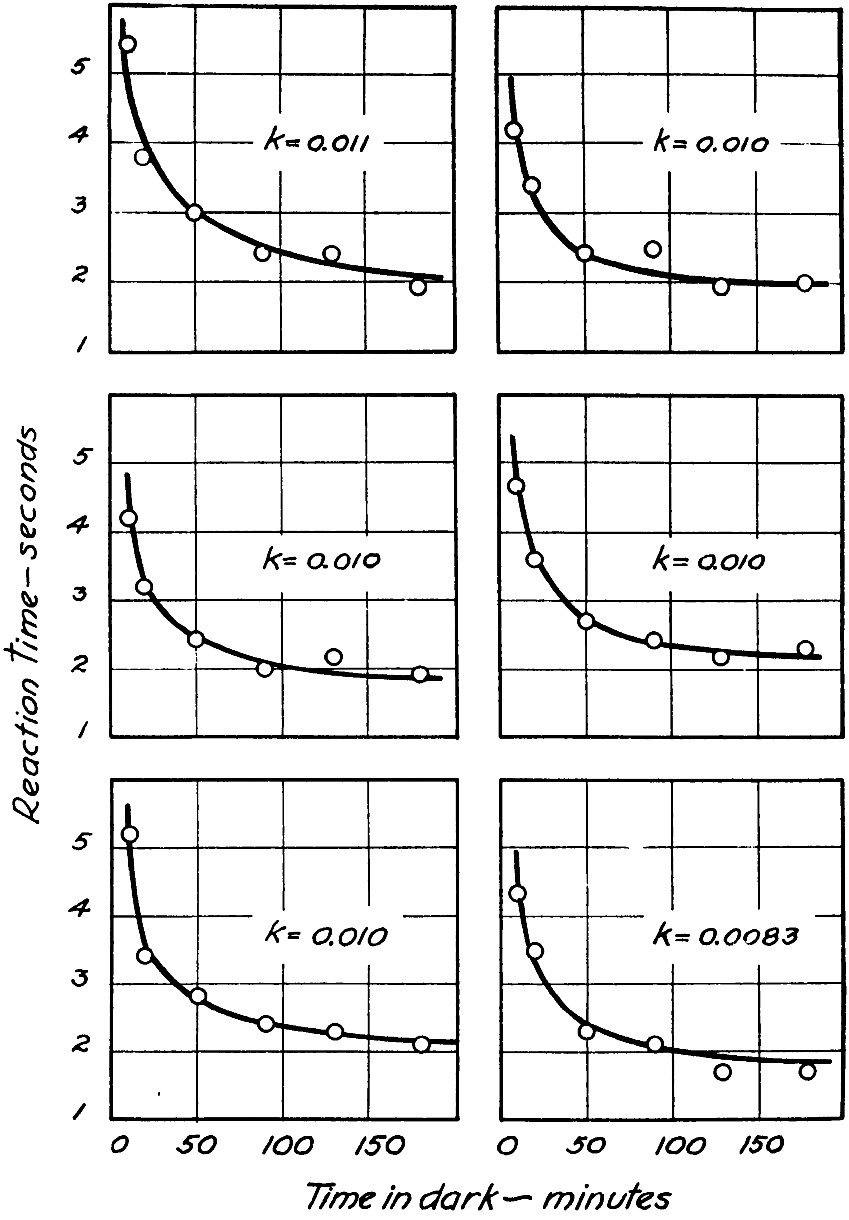


FIG. 5. Dark adaptation of *Ciona*. Each point is a single reading of the reaction time of a single animal. With each animal the points were secured in two runs of dark adaptation; the first, third, and fifth were made on 1 day, the remaining three on the following day. The curve in each case is calculated from the equation of a bimolecular reaction.

The dark adaptation of *Ciona* is a very slow process (*cf.* Hecht, 1918–19 *a*), and takes about 7 or 8 hours for completion. Most of the measurements were not carried on for that length of time because of the almost unbearable tediousness of the observations. Three series (VIII, IX*b*, and XV) were, however, carried on to 6, 7½, and 6 hours, respectively. The data secured are given in Tables V, VI, and VII, and in Fig. 6.

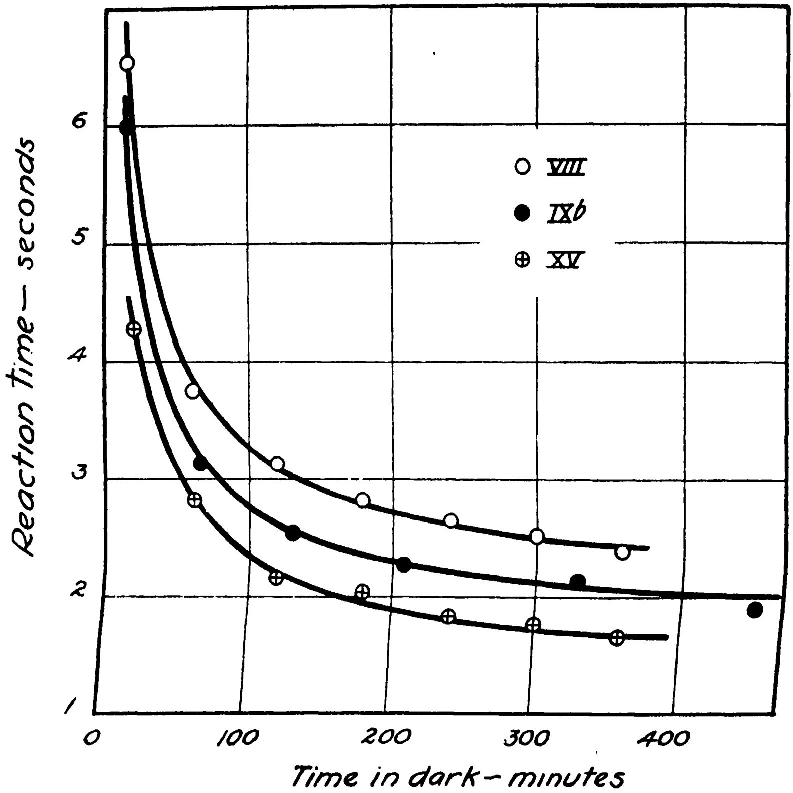


FIG. 6. Dark adaptation of *Ciona*. Series VIII represents 5 animals; Series IX*b*, 10 animals; and Series XV, 6 animals. The points for Series XV have been lowered 0.5 second in the drawing in order to keep them distinct from the others. The curves are all computed in terms of a bimolecular rection as explained in the text.

3. *Interpretation.*—The mathematical treatment of the data of

Ciona is the same as of *Pholas* and of *Mya*, with the slight exception that the upper limit of the total velocity, instead of being taken as the latent period velocity, is taken as the maximum reaction time velocity

TABLE V.

Dark Adaptation of Ciona. Series VIII. 5 Animals. Temperature, 15.8°C. Reaction Time at Complete Dark Adaptation Is 2.10 Seconds. $k = 0.0336$; $a = 0.385$.

Time in dark t	Reaction time r	
	Observed	$r = \frac{162.3 + 2.1\,t}{14.7 + t}$
min.	*sec.*	*sec.*
15	6.54	6.53
60	3.74	3.86
120	3.14	3.08
180	2.84	2.78
240	2.66	2.62
300	2.52	2.52
360	2.39	2.45

TABLE VI.

Dark Adaptation of Ciona. Series IXb. 10 Animals. Temperature, 17.1°C. Reaction Time at Complete Dark Adaptation (t = 16 Hours) Is 1.82 Seconds. $k = 0.0324$; $a = 0.472$.

Time in dark t	Reaction time r	
	Observed	$r = \frac{119.2 + 1.82\,t}{9.30 + t}$
min.	*sec.*	*sec.*
15	5.99	6.03
65	3.15	3.20
130	2.54	2.55
210	2.29	2.29
330	2.14	2.12
450	1.87	2.04

obtainable at complete dark adaptation. This, like the latent period, is also an experimentally determined value.

The data as with *Pholas* and *Mya* fall on the familiar hyperbola corresponding to a bimolecular reaction. The curves in Fig. 6 are all calculated in this way, and they show, as do the comparisons in Tables V, VI, and VII, that the agreement between calculation and experiment is very good.

TABLE VII.

Dark Adaptation of Ciona. Series XV. 6 Animals. Temperature, 16.5°C. Reaction Time at Complete Dark Adaptation Is 1.82 Seconds.
$k = 0.0268$; $a = 0.426$.

Time in dark	Reaction time r	
	Observed	$r = \frac{159.0 + 1.82\,t}{19.7 + t}$
min.	*sec.*	*sec.*
22	4.77	4.77
60	3.35	3.36
120	2.67	2.70
180	2.53	2.44
240	2.31	2.29
300	2.24	2.21
360	2.13	2.14

TABLE VIII.

Dark Adaptation of Frog Tadpole. Data from Obreshkove. 4 Animals. Latent Period, 1.0 Second. $k = 0.00205$.*

Time in dark t	Reaction time	
	Observed	Calculated
min.	*sec.*	*sec.*
10	52.3	52.3
20	30.0	28.7
30	20.6	20.6
40	15.2	15.3
50	12.8	12.8
60	12.7	10.8

* Obreshkove (1921), p. 268.

V.

Tadpoles.

1. Data.—In the course of some experiments on the photic responses of frog tadpoles, Obreshkove (1921) measured the dark adaptation of 4 animals. The results are so regular that they must surely be of theoretical interest. Obreshkove, however, merely presents the data without entering into a study of their meaning.

The tadpoles, when exposed to illumination, execute a specific reaction in the nature of a sudden forward movement after a definite reaction time. This response, curiously, is not mediated through the eyes, because removal of both eyes does not alter the sensibility of the animal either qualitatively or quantitatively.

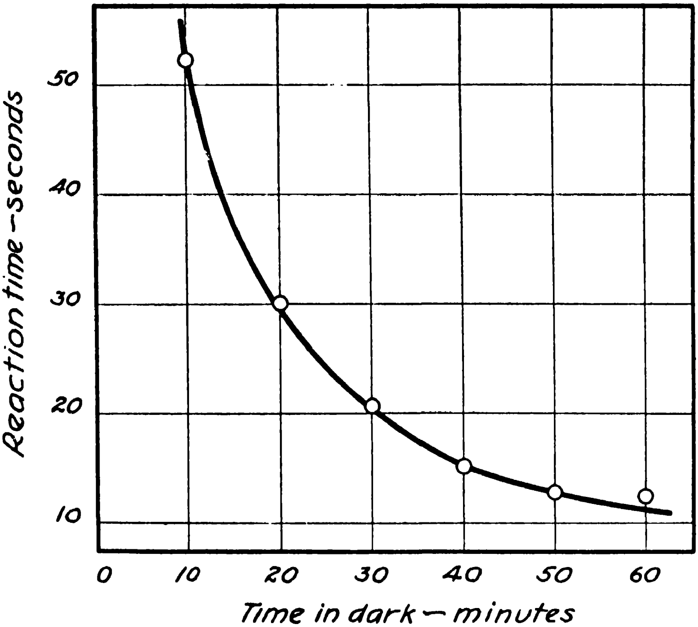


FIG. 7. Dark adaptation of frog tadpoles. Data from Obreshkove (1921). Each point is the average of 4 animals. The curve here again is calculated on the basis that dark adaptation depends on the kinetics of a bimolecular reaction.

The method of measuring dark adaptation employed by Obreshkove is identical with that described for *Ciona*, *Mya*, and *Pholas*. The animal is exposed to an illumination of 10 meter candles for 1 hour, after which it is placed in the dark and at regular intervals its reaction time to the same light of 10 meter candles is measured. The data are given in Table VIII and Fig. 7, in which each point is the average of four readings, one with each of 4 animals.

2. *Interpretation.*—The theoretical treatment of these data is identical with those of *Pholas* and *Mya.* Dark adaptation conforms to the kinetics of a bimolecular process. The agreement between experimental and calculated values is shown both in Table VIII and in Fig. 7, where the smooth curve is the course of the process computed in the same manner as with the other animals.

The actual handling of the data involves the difficulty that the latent period has not been measured accurately. Obreshkove recognized the existence of a latent period, and tried to determine its magnitude by measuring the reaction time to comparatively high intensities, when the reaction time is almost entirely latent period. For the reaction time to the maximum intensity (500 meter candles) he records an average of 0.76 second, which he treats as the upper limit for the latent period.

This datum must be considered doubtful for two reasons. In the first place the measurements were made with a stop-watch. This instrument is obviously incapable of recording accurately values below 1 second in the hands of any experimenter, because of the time lost from eye to hand, and from the beginning of the finger contraction to the releasing of the watch movement. The eye-to-hand time is about 0.2 second. If the starting lag of the stop-watch is added to this, it makes a period which cannot be neglected in stop-watch records of less than 1 second, and renders the value of such records very uncertain. In the second place, the average value of 0.76 second for the reaction time is apparently derived by the dubious process of not counting the values which are above 1.0 second. Obreshkove gives the 29 individual measurements for this particular intensity. Of them 22 are below 1.0 second; 7 are above. By omitting these 7 one gets 0.76 as an average. The average of all the experimental values, since there seems to be no reason for omitting any, is 0.94 second. Neither of these values is an accurate measure of the latent period. But they indicate that its duration is of the order of 1 second.

Fortunately this is all that is necessary. The values of the reaction time during dark adaptation are quite large: the longest is 52.3 seconds, and the shortest is 12.7 seconds. An error of even 0.5 second in the latent period introduces an error of 1 per cent and 4 per cent, respectively, in the maximum and minimum exposures. I have therefore assumed the latent period to be 1.0 second and have used this value in computing the figures in Table VIII and the curve in Fig. 7. Actually, if the latent period be assumed as 0.7 second or as 1.3 second it makes no noticeable difference in the computed values as given in this table and figure.

The data of the dark adaptation of the tadpole are therefore represented by the isotherm of a bimolecular reaction, and show the adequacy of the theoretical treatment to which they have been subjected.

VI.

CONCLUSION.

1. Reaction Order.—At first sight it may seem strange that the 3 animals here investigated and the 1 studied by Obreshkove should all show a dark adaptation whose kinetics correspond to the same order of reaction. This is not surprising, however, on intimate acquaintance with the animals, because the similarity of their photosensory make-up is apparent in a variety of ways: in their response to light; in the composition of their reaction time; in the effect of temperature on the two parts of their reaction time; etc. The identity of the order of the reaction underlying dark adaptation is thus only one more property which points to a basic similarity of the photosensory process in these species.

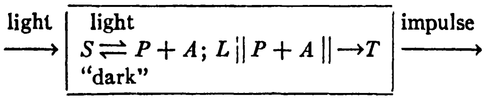
This must not be interpreted to mean that the actual substances entering into the reaction are identical in all the species. In fact, there is evidence—such as the specific spectral sensitivity, and the specific effects of temperature, to be published shortly—which shows that these substances are not the same. It is merely their arrangement and the order of their reaction—in short their organization—which seem to be identical in the different animals.

That the reaction underlying dark adaptation is bimolecular and not monomolecular is not unexpected. Dark adaptation is a process in the course of which a sensitive material accumulates in the sense cell as the result of a chemical reaction. If one supposes that the sensitive material is built up mainly from the products of its photolysis, then a bimolecular reaction is almost the simplest process conceivable.

2. *Photosensory Mechanism.*—The idea of such a reversible system has been at the basis of all the work which has grown out of the original experiments with *Mya*, and which in turn have served to define that system more rigorously.

It is supposed that a photosensitive material, S, is decomposed by light into at least two substances, P and A, which at the same time tend to recombine and form the original material, S. This reversible reaction has been referred to as the primary photochemical reaction. By itself it is sufficient to account for only a part of the characteristics of the photosensory process in these animals. The existence of a latent period whose duration is dependent on the primary photochemical reaction calls for an additional process in the sense cell. An inactive substance, L, is converted into an active form, T, a definite accumulation of which sets off, electrically or chemically, the nerve-ending attached to the sense cell and thus starts the train of events culminating in the specific response of the organism. This latent period reaction $L \rightarrow T$ can proceed only in the presence of P and A freshly formed from S by the primary photochemical reaction. This relation is probably a simple catalysis.

In a diagrammatic way these two interrelated reactions may be written



$$\xrightarrow{\text{light}} \boxed{S \underset{\text{“dark”}}{\overset{\text{light}}{\rightleftharpoons}} P + A;\ L \,\|P + A\|\, \rightarrow T} \xrightarrow{\text{impulse}}$$

where $\|P + A\|$ means catalysis by $P + A$. The rectangle represents the sense cell. Light comes in at one end and the impulse for the nerve leaves the other end. The two reactions are given here as separated in space; it is to be understood that they are intimately mixed in the cell.[5]

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[5] The following statement occurs in a recent paper by Folger (1926, p. 368). "*Mast ('07) was among the first to postulate a reversible photochemical process to account for photic response in lower organisms. Hecht ('19) elaborated this idea to explain the response to light in Mya arenaria.*" In order to make clear the developmental sequence of the ideas here involved, the complete statement of Mast's hypothesis, taken from the paper referred to (Mast, 1907, p. 159), is given verbatim. "*To explain reversal in the sense of reaction on the basis of chemical reactions induced by light let us assume: (1) That Volvox contains substances X and Y,*

The primary reaction, here written as a completely reversible reaction

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A \tag{13}$$

may indeed by only a pseudoreversible reaction

$$\begin{array}{l} \text{light} \\ S \longrightarrow P + A + B \\ S \longleftarrow P + A + C \\ \text{"dark"} \end{array} \tag{14}$$

in which B is the substance that actually controls the reaction $L \rightarrow T$, and C is a substance present in excess. In picturing concretely the behavior of such a system as given in the above rectangular diagram the pseudoreversible reaction is often more helpful than the simpler, completely reversible one. Mathematically, the two are identical.

Such a coupled system of two reactions describes most of the features exhibited by the photosensory process, not merely qualitatively but in quantitative detail as well. It accounts for the composition of the reaction time; for the different effects of temperature on the two parts of the reaction time; and for the interrelation of the two parts of the reaction time. It accounts for the spectral sensitivity of the animals; for the kinetics of dark adaptation, as we have seen; and for the effect of temperature on dark adaptation. It accounts for light adaptation and sensory equilibrium; for the sensitivity changes at different light levels; and for intensity discrimination. It even includes the proof of the major assumption made in the theoretical treatment of dark adaptation, namely that under all conditions of adaptation a constant photochemical effect is necessary

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chemical reaction between which is regulated by the intensity of light; (2) that a suboptimum intensity favors the formation of substances represented by X and a supraoptimum intensity those represented by Y; and (3) that the colonies are neutral in reaction when there are Y substances in one member of the equation and X in the other; positive when one member contains (X+) substances and the other (Y−), and negative when one contains (X−) and the other (Y+)." Probably the first use of the idea of a reversible photochemical reaction in relation to the photosensory process was made by Müller (1896).

to produce a definite response in the animal. The general idea involved therefore leads to a consistent and rational view of the nature of the photosensory process.

VII.

SUMMARY.

1. Data are presented for the dark adaptation of four species of animals. They show that during dark adaptation the reaction time of an animal to light of constant intensity decreases at first rapidly, then slowly, until it reaches a constant minimum.

2. On the assumption that at all stages of adaptation a given response to light involves a constant photochemical effect, it is possible to describe the progress of dark adaptation by the equation of a bimolecular reaction. This supposes, therefore, that dark adaptation represents the accumulation within the sense cells of a photosensitive material formed by the chemical combination of two other substances.

3. The chemical nature of the process is furthei borne out by the fact that the speed of dark adaptation is affected by the temperature. The velocity constant of the bimolecular process describing dark adaptation bears in *Mya* a relation to the temperature such that the Arrhenius equation expresses it with considerable exactness when $\mu = 17{,}400$.

4. A chemical mechanism is suggested which can account not only for the data of dark adaptation here presented, but for many other properties of the photosensory process which have already been investigated in these animals. This assumes the existence of a coupled photochemical reaction of which the secondary, "dark" reaction is catalyzed by the products of the primary photochemical reaction proper. This primary photochemical reaction itself is reversible in that its main products combine to form again the photosensitive material, whose concentration controls the behavior of the system during dark adaptation.

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HYDRATION OF GELATIN IN SOLUTION.

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The high viscosity of gelatin solutions indicates that even at temperatures as high as 40°–50°C. gelatin is hydrated,[1] *i.e.*, that the ultimate gelatin units (particles or molecules) in the solution are in some way combined with a certain amount of water. This is true not only in the case of ionized gelatin, where the hydration is regulated by the Donnan equilibrium, but also in the case of isoelectric gelatin, the viscosity of which, even at high temperatures, is quite high as compared with that of crystallized egg albumin of the same concentrations, as shown in Fig. 1. The hydration of gelatin is indicated also by measurements of osmotic pressure of various concentrations of gelatin solutions. In this respect gelatin differs from egg albumin. The curve, Fig. 2, for osmotic pressure *vs.* concentration for isoelectric albumin (sp. cond. about 8×10^{-5} in 15 per cent sol.) at 20°C. is a straight line, while in the case of gelatin the osmotic pressure increases much faster than the concentration, and the curve is convex toward the concentration axis. This difference in the behavior of gelatin and egg albumin with respect to osmotic pressure is explainable by the difference in their degree of hydration. As was shown elsewhere,[2] the osmotic pressure of dilute molal solutions of hydrated substances may be expressed as

$$P = K \frac{C}{100 - \varphi} \qquad (1)$$

where $K = \frac{RT}{M}$ (M = mol weight of solute).

C = gm. of solute per 100 cc. of solution.

φ = volume of the hydrated solute, which means that the osmotic pressure would be proportional to the concentration if the concentration were corrected for the volume occupied by the *hydrated* solute.

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[1] Bogue, R. H., The chemistry and technology of gelatin and glue, New York and London, 1922, 194.

[2] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 723.

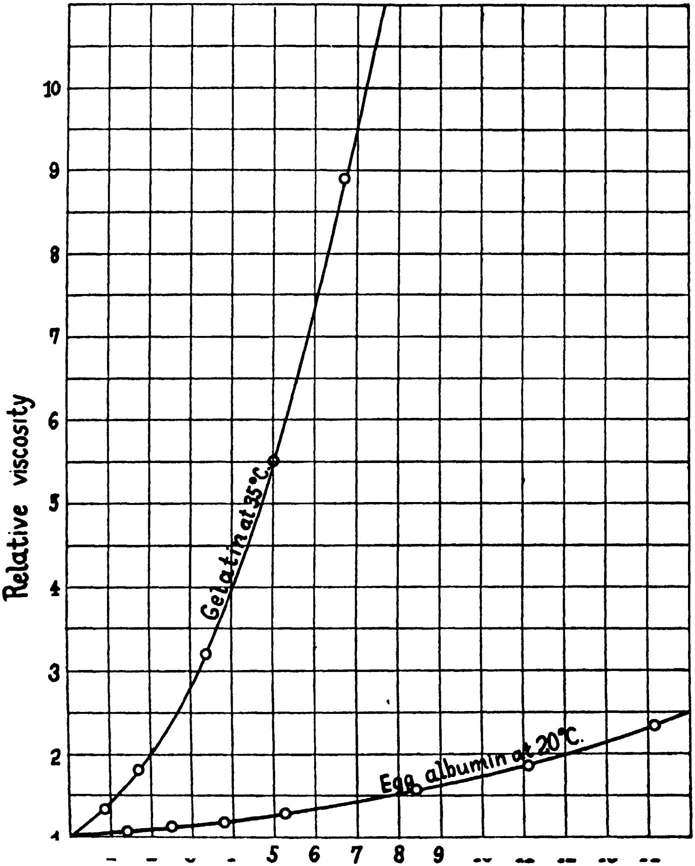

FIG. 1. Viscosity-concentration curves of isoelectric gelatin at 35°C. and of isoelectric egg albumin at 20°C.

In the case of egg albumin the correction is small, and if C is expressed in gm. per 100 cc. of H_2O, there is then practically no correction, and the plotted curve is a straight line. In the case of gelatin, on the other hand, the volume occupied by the hydrated solute is quite large, so that the active concentration of gelatin as expressed in gm.

per 100 cc. of H_2O is much greater than the one taken from dry weight measurements. Hence the osmotic pressure increases much faster than the dry weight concentration. The curves, as said before, should become straight lines if the concentrations of the gelatin solutions were corrected for the volume of the hydrated solute. A means of finding this correction is afforded by viscosity measurements.

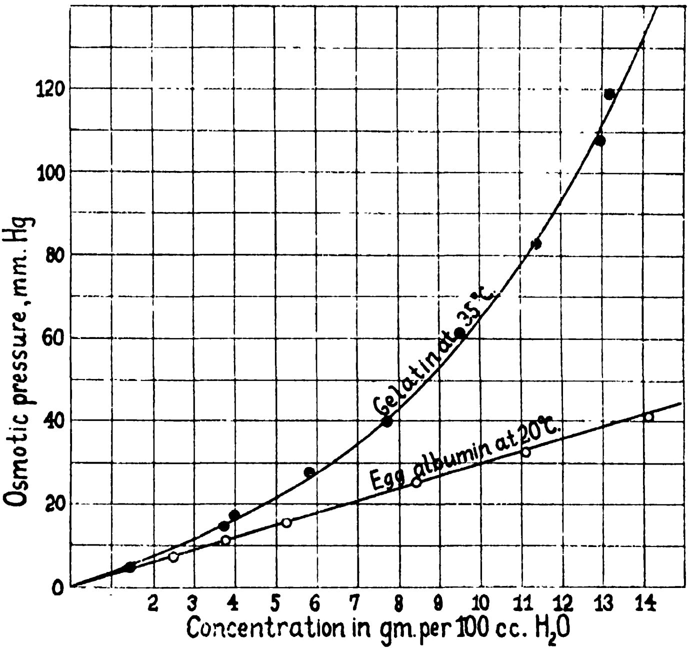

FIG. 2. Osmotic pressure-concentration curves of isoelectric gelatin at 35°C. and of isoelectric egg albumin at 20°C.

A Method for Measuring Hydration of Gelatin.

In a recent publication,[3] the writer showed that the viscosity of a number of colloidal solutions, as well as of various sugar solutions, as measured by means of an Ostwald viscosimeter, may be well represented by the equation

$$\frac{\eta}{\eta_0} = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^4} \tag{2}$$

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[3] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 715.

where η is the absolute viscosity of the solution, η_0 is the absolute viscosity of the solvent, and φ is the volume occupied by the solute expressed as a fraction of the total volume of the solution. In the case of sugars or other substances that are hydrated to a very small extent, the value of φ when expressed as cc. per 100 cc. of solution actually equals the volume of the dissolved dry substance, while in the case of highly hydrated or solvated substances, such as casein in water or rubber in benzene, φ represents the volume of the dry substance plus the volume of the solvent associated with it. This was checked for rubber by substituting the values for φ as obtained from viscosity measurements into the equation for osmotic pressure, (equation (1)). The values of K for various concentrations of rubber were constant. The data of Caspari[4] were used in both cases.

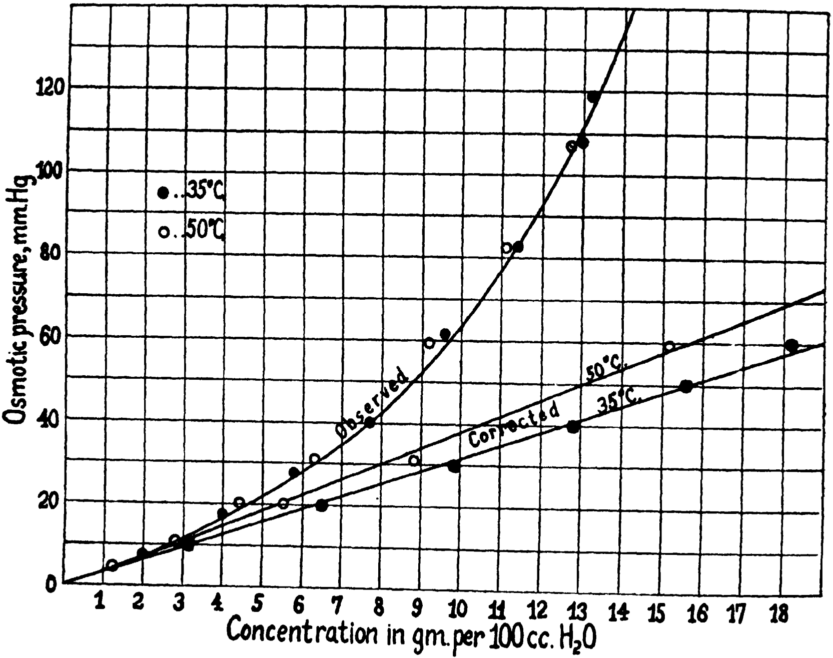

FIG. 3. Effect on the osmotic pressure-concentration curves of gelatin of correcting the concentration of the gelatin for the water of hydration as calculated from viscosity measurements.

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[4] Caspari, W. A., *J. Chem. Soc.*, 1914, cv, 2139.

This method of testing the validity of the viscosity formula is applied in this paper to isoelectric gelatin. A series of curves for the osmotic pressure of various concentrations of isoelectric gelatin at various temperatures were plotted. All the curves showed the characteristic convexity toward the concentration axis. The concentrations were expressed as gm. of dry gelatin per 100 cc. H_2O, as determined by drying for 24 hours at 100°C., *definite weights* of gelatin solution taken from the osmometers after equilibrium was reached. At the same time viscosity measurements were carried out by means of an Ostwald viscosimeter on samples of the same gelatin solutions at the same temperatures at which the osmotic pressure measurements were done. The viscosities of freshly prepared gelatin solutions were also measured and gave practically identical results. The various values of φ were then read off from the theoretical viscosity curve shown elsewhere,[5] and the concentrations in the osmotic pressure curves were then corrected. Fig. 3 shows that the corrected points for the osmotic pressure values lie on straight lines in the range of the dry weight concentration from 1 to 10 gm. per 100 cc. of H_2O for the various temperatures used, thus proving that the values of φ as obtained from the viscosity represent the true volumes of the hydrated gelatin particles, and hence affording a method of determining the degree of hydration of gelatin.

Molecular Weight of Gelatin.

One of the difficulties usually experienced in calculating the mol weight of gelatin from osmotic pressure through the application of van't Hoff's formula

$$P = \frac{RT}{M} C \ (C = \text{gm./cc.}),$$

is the fact that P/C, as usually plotted, is not constant. But if, on the other hand, the concentration is corrected for the "water of hydration" by means of viscosity, then RT/M becomes a constant value, expressed as the slope of the corrected curve, or it can be calculated more exactly from the relation

$$\frac{RT}{M} = \frac{P \times (100 - \varphi)}{C} \tag{3}$$

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[5] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 717.

At 35°C. the value of RT/M is 313, as shown in Table I.

Hence

$$M = \frac{RT}{313}$$

R being the gas constant equals

$$\frac{P_0\,V_0}{T_0} = \frac{22.4 \times 760 \times 1000}{273} = 62{,}400 \text{ cc.mm.Hg/degree}$$

or

$$M = \frac{62{,}400 \times 308}{313} = 61{,}500.$$

TABLE I.

C	$\frac{\eta}{\eta_0}$	φ	Corrected concentration $= \frac{C}{100-\varphi}$	P	$\frac{RT}{M}$ uncorrected $= \frac{100\,P}{C}$	$\frac{RT}{M}$ corrected $= \frac{(100-\varphi)\,P}{C}$
gm./100 cc. of solution				*mm.Hg*		
1	1.43	7.75	1.08	3.5	350	324
2	2.06	15.05	2.35	7.5	375	319
3	2.96	21.80	3.84	12.0	400	312
4	4.24	27.90	5.55	17.0	425	306
5	6.00	33.40	7.50	23.0	460	307
6	8.20	38.10	9.70	29.5	492	304
7	10.85	42.18	12.1	37.5	537	310
8	13.9	45.52	14.7	47.0	588	320
Average..						313

This value represents the weight of dry gelatin which, if dissolved in 1000 cc. of H_2O, will produce an osmotic pressure of $22.4 \times \frac{308}{273}$ atmospheres at 35°C.

The Mechanism of Hydration of Gelatin.

The experiments of Loeb[6] on viscosity of gelatin at various pH led him to the conclusion that gelatin solutions contain a number of submicroscopic particles of solid jelly which are able to take up water and swell when there is a greater osmotic pressure inside the particles than outside. On the addition of acid or alkali to a solution of isoelectric gelatin a Donnan equilibrium is established between the diffusible ions inside and outside of the particles with the result that, owing to the larger concentration of gelatin inside, there are more ions inside than outside, and the particles then increase in volume on account of the difference in osmotic pressure. According to this view, then, these submicroscopic particles of solid jelly behave with respect to the Donnan equilibrium in agreement with the Procter-Wilson[7] theory of swelling of blocks of gelatin. According to this theory a block of gelatin under the influence of a higher ion activity inside than in the surrounding medium takes up water until the difference in the osmotic pressure between the inside and the outside solution is balanced by the stress in the elastic structure of the block, which appears to obey Hooke's law, *i.e.*,

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[6] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924, 270.

$$e = E\,V$$

where e is the osmotic pressure due to the difference in the activity of ions, E is the bulk modulus, and V is the increase in volume.

This theory appears to hold also for the swelling of blocks of *isoelectric* gelatin, as was shown by Northrop and the writer.[8] In the case of isoelectric gelatin, where ions are practically absent, an osmotic pressure exists in a block of solid gelatin due to the presence of a water-soluble constituent of gelatin held in a network of insoluble fibers. This was confirmed by actually isolating from gelatin, by fractional precipitation with alcohol, two fractions, one of which is soluble in cold water, does not set to a jelly, and has a low viscosity and a high osmotic pressure, and a second one which is insoluble at ordinary temperatures, sets to a jelly in very low concentration, and swells much less than ordinary gelatin. When a block of ordinary solid isoelectric gelatin is immersed in water, the water enters the gelatin which swells until the osmotic pressure of the soluble fraction of the gelatin inside of the block is equal to the elasticity pressure of the block, *i.e.*,

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[7] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Wilson, J. A., and Wilson, W. H., *J. Am. Chem. Soc.*, 1918, xl, 886.

[8] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161.

$$P = E\frac{V_s - V_0}{V_0},$$

where P is the osmotic pressure in the block which can be measured directly as described,[9] E is the bulk modulus of elasticity, V_S is the final volume of the block at equilibrium, and V_0 is the volume of the dry gelatin.

It will now be shown from the viscosity measurements of various concentrations of isoelectric gelatin at 35°C. that the apparent hydration of gelatin at this temperature is due to the swelling of the ultramicroscopic particles of solid jelly brought about by the same mechanism as the swelling of large blocks of gelatin,—namely, by the osmotic pressure of the *soluble* gelatin which is included in the insoluble particles of solid jelly, or micellæ as they were named by Naegeli. The particles swell until the difference between the osmotic pressure inside and outside of the particles is balanced by the elastic pressure of the particle.

Let q be amount of water in cc. held by 1 gm. of gelatin, as calculated from viscosity, *i.e.*

$q = \frac{\varphi}{C} - .75$, the last value being the volume of 1 gm. of dry gelatin.

Let also n be the number of micellæ per gm. of gelatin.

$\frac{q}{n}$ = cc. H_2O per micella, under the assumption that all the water of hydration is associated with the micellæ only.

s = gm. of soluble gelatin per micella.

K = osmotic pressure constant for soluble gelatin, *i.e.*,

$$K = \frac{RT}{M}$$

Then the osmotic pressure inside at equilibrium equals $\frac{Ks}{q/n} = \frac{Ksn}{q}$. The osmotic pressure outside is the osmotic pressure P as determined directly, while the elastic pressure is

$$E\frac{q/n}{v_0} = E\frac{q}{v_0 n}$$

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[9] Northrop and Kunitz,[8] p. 162.

where E is the bulk modulus of the micellæ at the given temperature, and v_0 is the volume of the micella before swelling. The equilibrium condition is then

$$\frac{Ksn}{q} - P - E\frac{q}{v_0 n} = 0;$$

or if instead of sn the symbol α is used, where α designates the fraction of 1 gm. of gelatin which is found in a soluble state inside of the micellæ, then the equation becomes

$$\frac{K\alpha}{q} - P - E_1 q = 0, \tag{4}$$

where

$$E_1 = \frac{E}{v_0 n}$$

As the concentration of the gelatin approaches zero, P approaches it likewise, and we have then

$$\frac{K\alpha}{q} - E_1 q = 0$$

$$[P \doteq 0]$$

or

$$\frac{K\alpha}{E_1} = q^2$$

$$[P \doteq 0]$$

If a curve is plotted for the values of q as obtained from viscosity measurements against the concentrations of gelatin used and if the curve is continued until it crosses the q axis, the value of q at the interception may then be introduced into the last equation. The actual value of q^2 as read off from the curve is close to 52. Thus we have

$$\frac{K\alpha}{E_1} = 52.$$

When P is not equal to zero the equilibrium equation becomes

$$\frac{52}{q} - \frac{P}{E_1} - q = 0 \tag{5}$$

or

$$\left(\frac{52}{q} - q\right) + P = \frac{1}{E_1} \tag{5a}$$

The following table shows the value of $1/E_1$ for various concentrations of isoelectric gelatin at 35°C.

TABLE II.

C	P	q	$\frac{1}{E_1} = \left(\frac{52}{q} - q\right) + P$
gm./100 cc. of solution	*mm. Hg*		
1	3.5	7.00	.123
2	7.5	6.78	.120
3	12.0	6.52	.123
4	17.0	6.30	.125
5	23.0	5.93	.123
6	29.5	5.60	.125
7	37.5	5.28	.122
8	47.0	4.94	.120
Average...			.123

Substituting into equation (5) for $1/E_1$ its value of .123, and for P the relation

$$P = \frac{K_1 C}{100 - \varphi} \text{ (equation (3)), where}$$

$$K_1 = 313^* \text{ and } \varphi = (q + .75)C$$

we get

$$\frac{52}{q} - \frac{38C}{100 - (q + .75)C} - q = 0 \tag{6}$$

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* Table I.

Fig. 4 shows the plotted theoretical curve for equation (6), obtained by assuming various values for q and solving for C. On the same figure are also shown the experimental values for q at various values of C, which are very close to the theoretical line. This agreement between the theoretical values of the water of hydration of gelatin with the values obtained from viscosity measurements confirms the theory that a solution of isoelectric gelatin even at 35°C. contains a definite number of small blocks of gelatin filled with a definite weight of soluble gelatin the osmotic pressure of which is higher than the osmotic pressure of the gelatin outside of the blocks, *i.e.* higher than the total osmotic pressure of the gelatin solution as a whole. Owing to this difference in pressure, each little block swells until its osmotic pressure is balanced by the total osmotic pressure of the solution and the elastic resistance of the block to stretch.

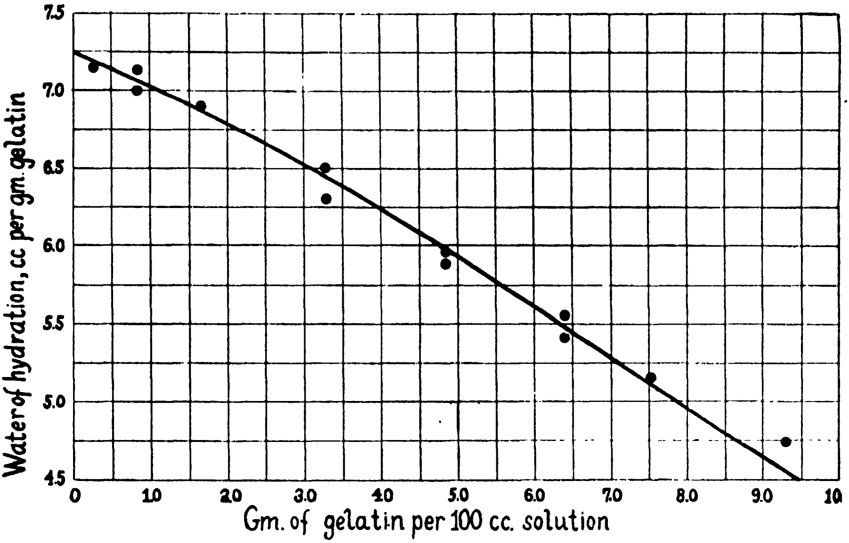

FIG. 4. Relation between the concentration of gelatin and the amount of water of hydration per gm. of dry gelatin. The dots are the values obtained from viscosity measurements. The smooth curve represents the theoretical relation:

$$\frac{52}{q} - \frac{38C}{100 - (q + .75)C} - q = 0.$$

As the concentration of the gelatin is increased the total osmotic pressure of the solution is increased. The number of little blocks is also increased, but the amount of soluble gelatin per block is unchanged, with the result that the difference in osmotic pressure between the inside and the outside of the block continuously decreases with the increase in the total concentration of the gelatin solution. The swelling of the little blocks is thus decreasing gradually as shown on the curve. This is also clear from equation (5*a*) where $\frac{52}{q} - q = .123\ P$. An increase in P must be followed also by an increase in the value of $\frac{52}{q} - q$, which is possible only when q is diminishing in its value.

The Quantitative Interpretation of pH Viscosity Curves.

If the equation $\frac{K\alpha}{q} - P - E_1\,q = 0$ is true for isoelectric gelatin then at any other pH outside of the isoelectric point the equation should be $\frac{K\alpha}{q} - P + D.P. - E_1\,q = 0$ where P equals

$$\frac{313C}{100 - (q + .75)C}$$

and $D.P.$ is the difference between the osmotic pressure inside and outside of the micellæ due to the difference in the ion activity brought about by the Donnan equilibrium established between the micellæ and the gelatin solution outside of the micellæ. This will be true only if the addition of acid or alkali does not modify the values of α and E_1. That this is the case for HCl, at least, was demonstrated by the fact of the reversibility of the swelling of gelatin in an HCl solution, since on washing away the HCl the swollen gelatin returns to the original volume of isoelectric gelatin.[10] This is also true with respect to osmotic pressure. Hence, if values could be substituted for K, α, and E_1, then it would be possible to calculate the internal Donnan pressure of gelatin at various pH from the viscosity measurements. But, as found above,

$$\frac{K\alpha}{E_1} = 52$$

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[10] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–27, viii, 329.

and

$$\frac{1}{E_1} = .123 \quad \text{or} \quad E_1 = 8.1$$

Hence

$$K\alpha = 420$$

Substituting these values, the equation for the internal Donnan pressure of a solution of gelatin chloride at various pH is then

$$D.P. = P + 8.1\,q - \frac{420}{q} \tag{7}$$

There is also another way of obtaining the approximate values of the internal Donnan pressure of the micellæ; namely, through an analysis of the ion distribution between the micellæ and the outside gelatin solution.

Let H_i = hydrogen ion activity in the micellæ.
$[HCl_i]$ = total HCl concentration in the micellæ.
H_0 = hydrogen ion activity outside.
$[HCl_0]$ = total HCl concentration outside.

If it is to be assumed that the gelatin has only a slight effect on the activity of the Cl ion,[11] and that the concentration of HCl outside of the micellæ is not much different than the average concentration of HCl in the gelatin solution, then according to the Donnan equilibrium relation

$$H_i\,\gamma_{Cl_i}\,[HCl_i] = H_0\,\gamma_{Cl_0}\,[HCl_0]$$

where γ_{Cl_i} and γ_{Cl_0} are the activity coefficients for Cl ion,

or
$$H_i\,[HCl_i] = H_0\,[HCl_0]\,\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} \tag{8}$$

The calculations of Loeb[12] on osmotic pressure of gelatin have shown that the Donnan osmotic pressure of a solution of gelatin in a collodion membrane is equal approximately to the sum of the activities of the ions in the gelatin solution, as measured electrometrically, multiplied by $RT/1000$. This same pressure is also acting against the Donnan pressure of the micellæ, which in its turn equals the sum of the activities of the ions in the micellæ. Hence the difference in the Donnan pressure is

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[11] Loeb,[6] p. 50. Hitchcock, D. I., *J. Gen. Physiol.*, 1922–23, v, 387. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 34.

[12] Loeb,[6] p. 217.

$$D.P. = \frac{RT}{1000} (H_i \times Cl_i - H_0 - Cl_0) \tag{9}$$

where

$$Cl_i = \gamma_{Cl_i} [HCl_i] \text{ and } Cl_0 = \gamma_{Cl_0} [HCl_0].$$

Equation (8) may also be put in the form of

$$H_i \left(\frac{H_i}{\gamma_{H_i}} + [HCl_c]\, C \right) = \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}\, a \tag{10}$$

where $[HCl_c]$ = equivalent concentration of HCl combined with 1 gm. of gelatin when dissolved in 100 cc. of solvent, C = concentration of gelatin in gm. per 100 cc. H_2O, and $a = H_0[HCl_0]$

or

$$H_i^2 + H_i\, [HCl_c]\, \gamma_{H_i}\, C = \gamma_{H_i} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}\, a \tag{10a}$$

If it is to be assumed, as a first approximation, that

$$\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} = 1$$

and that

$$\gamma_{H_i} = \gamma_{H_0}$$

then

$$H_i^2 + H_i\, [HCl_c]\, \gamma_{H_0} C = \gamma_{H_0}\, a \tag{10b}$$

or

$$H_i^2 + H_i\, [HCl_c]\, C_1 = a_1$$

where

$$C_1 = \gamma_{H_0}\, C \text{ and } a_1 = \gamma_{H_0}\, a$$

It will be shown later that of every gm. of gelatin dissolved at 35°C., 0.48 gm. is found in the micellæ. Hence if q is the amount of H_2O of hydration per gm. of gelatin, as obtained from viscosity measurement, the concentration of the gelatin in the micellæ, is $C = \frac{.48}{q}$, since the water of hydration is associated, according to theory developed here, with the micellæ only.

At any pH the amount of HCl combined per gm. of gelatin in the micellæ can be obtained from the titration curve of gelatin, since it was shown by experiment that there is practically no difference in the titration curves of the soluble and the insoluble fractions of gelatin. Fig. 5 shows the titration curve for 1 per cent solution of gelatin with HCl. The curve was corrected, from the data in Table IV, for the free HCl, *i.e.* for the HCl required to bring H_2O to the corresponding pH. At any value of H_i the value of HCl_c is thus determinable by reading it off the curve. The correction for the free HCl was based on the relation $[HCl] = \frac{H}{\gamma_H} + [HCl_c]C$ where

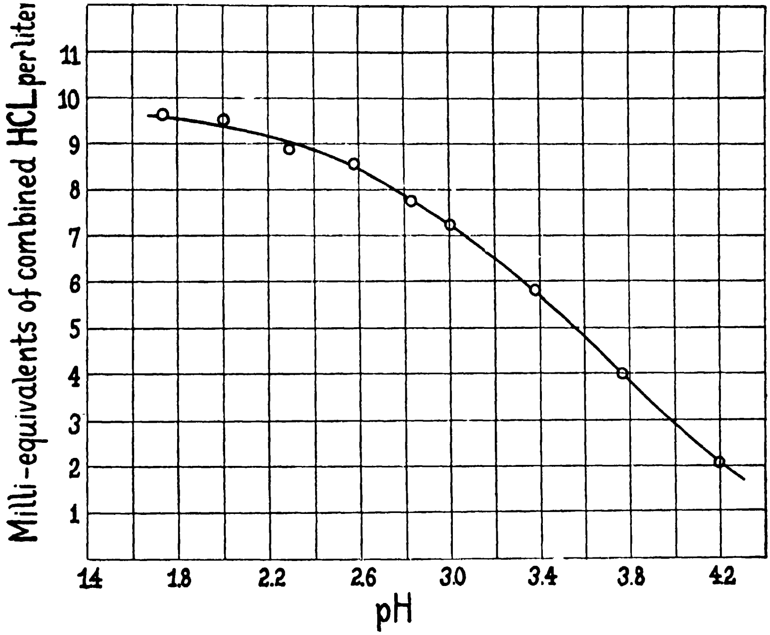

FIG. 5. Corrected titration curve of 1 per cent isoelectric gelatin with HCl.

[HCl] = total concentration of HCl in the gelatin solution.
H = activity of the H ion as obtained from pH measurement.
γ_H = activity coefficient of H at the ionic strength equal to [HCl].*
$[HCl_c]$ and C are the same as defined before.

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* Gelatin appears to affect neither the ionic strength of a solution nor the activity coefficients of the various ions. See Northrop and Kunitz,[11] p. 29.

Values for γ_H (as well as for γ_{Cl_o}, γ_{H_i}, and γ_{Cl_i}, used in the later calculations) at the various values of [HCl] were calculated, by the method of Lewis and Randall,[13] from the recent data of Scatchard[14] on the mean activity coefficients of KCl and HCl at 25°. The curves for γ_H and γ_{Cl} at various concentrations of HCl are shown on Fig. 6. All pH measurements were done electrometrically at 35°C., and the values for pH were based on 0.100 M HCl as a standard, its pH being taken as 1.085 at 35°. It is thus possible, by means of equation (10*b*), to calculate approximately the activities of the ions in the micellæ from the known data on the whole solution and from the additional information on the concentration of the gelatin in the micellæ from the viscosity measurements, especially since [HCl_c]

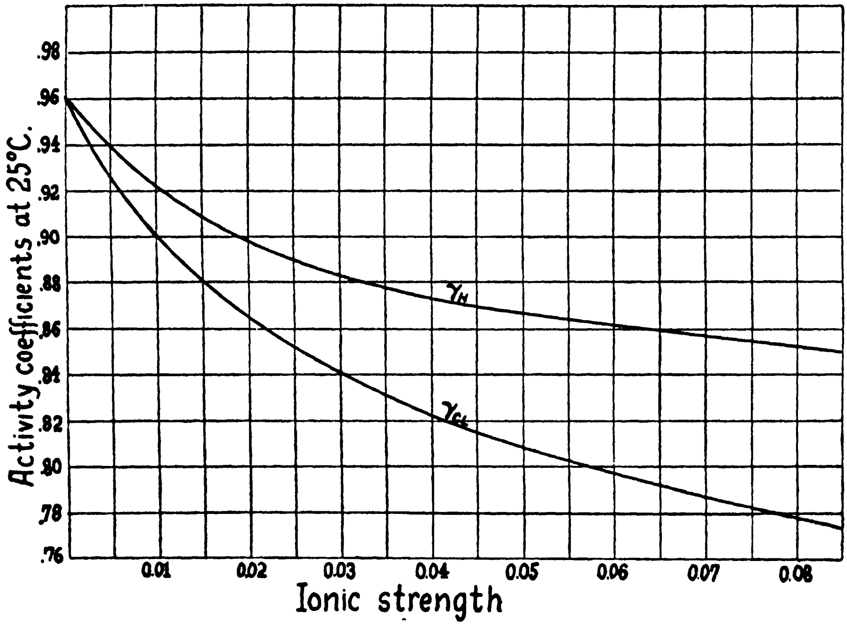

FIG. 6. Activity coefficients for hydrogen and chloride ions at various values of the ionic strength as calculated from the data of Scatchard on the mean activity coefficients of KCl and HCl at 25°C.

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[13] Lewis, G. N., and Randall, M., Thermodynamics and the free energy of chemical substances, New York and London, 1923, 381.

[14] Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 660.

can be easily expressed as a function of H_i by means of the equilibrium equation representing the titration curve. The actual calculations were done graphically by assuming various values of H and solving for a_1 at a given value of C_1. This was repeated for the identical values of H and another value of C_1. A family of curves were then plotted for C_1 of 2.0, 2.5, 3.0, 3.5, and 4.0 of pH as abscissæ and values of a_1 as ordinates. These curves were then used for finding the pH of the micellæ for various values of $\gamma_{H_0} a$ and $\gamma_{H_0} C$, thus giving values of H_i. On substituting the found value of H_i in equation (8), and assuming that $\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} = 1$, the value of $[HCl_i]$ was obtained, namely $[HCl_i] = \frac{a}{H_i}$. Using the obtained approximate values of $[HCl_i]$ as the ionic strength of the solution in the micellæ, values for γ_{H_i} and γ_{Cl_i} were

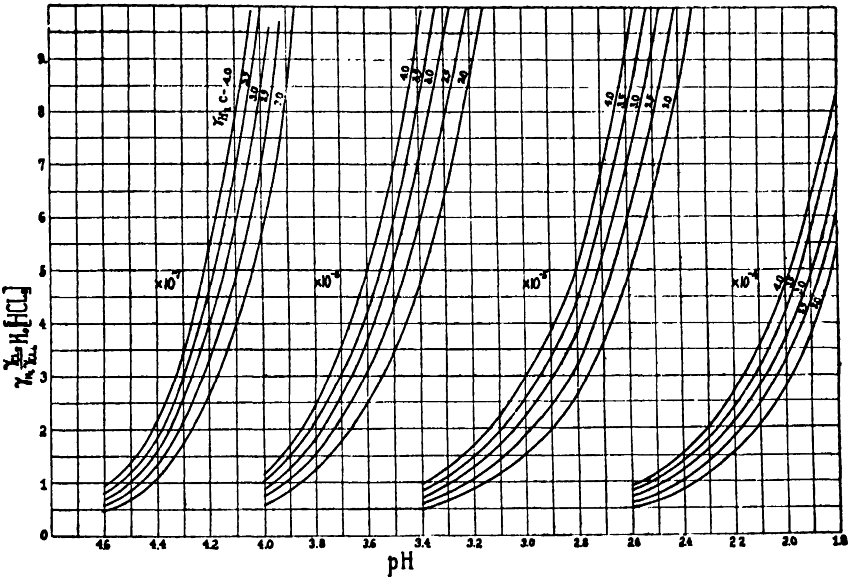

FIG. 7. Auxiliary curves for obtaining the values of pH in the micellæ of 1 per cent gelatin-HCl solution from the relation:

$$H_i^2 + H_i [HCl_i] \gamma_{H_i} C = \gamma_{H_i} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} H_0 [HCl_0]$$

read off the curves. New values of H_i and $[HCl_i]$ were then obtained in the same manner as before except instead of $a_1 = \gamma_{H_0}\, a$ and $C_1 = \gamma_{H_0} C$, values of $a_{\prime\prime} = \gamma_{H_i} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}\, a$ and $C_{\prime\prime} = \gamma_{H_i} C$ were employed; also in equation (8) the actual values of $\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}$ were used. The new values of H_i and $[HCl_i]$ happened to be almost identical with those obtained on the first approximation and hence they were taken as the correct ones. The sum of the activities of the ions inside of the micellæ is then $H_i + \gamma_{Cl_i} [HCl_i]$ and outside $H_0 + \gamma_{Cl_0} [HCl_0]$, and the Donnan pressure is

$$19.2 \times 10^3 \times \left(H_i + \gamma_{Cl_i} [HCl_i] - H_0 - \gamma\ [HCl_0]\right)$$

where 19.2×10^3 is the theoretical osmotic pressure of a molar solution in mm. of Hg at 35°C. The following is an example of the calculations.

A solution of 0.97 per cent gelatin containing 8.13 cc. N/10 HCl per 100 cc. gave a pH reading of 3.01. Its relative viscosity at 35°C. was found to be 2.50 with a value for q of 18.65.

$$[HCl_0] = 8.13 \times 10^{-3}$$

$$\left.\begin{aligned} \gamma_{Cl_0} &= .910 \\ \gamma_{H_0} &= .928 \end{aligned}\right\} \text{At ionic strength of } 8.13 \times 10^{-3}$$

$$H_0 = 9.77 \times 10^{-4} \text{ from pH}$$

$$a = H_0 [HCl_0] = 7.94 \times 10^{-6} \text{ and } \gamma_{H_0} a = 7.36 \times 10^{-6} = a_1$$

$$C = \frac{.48}{18.65} = 2.57 \text{ gm./100 cc. } H_2O \text{ and } \gamma_{H_0} C = 2.39 = C_1$$

pH inside (from curve for $C_1 = 2.5$ and extrapolated for 2.39) = 3.31

$$H_i \text{ from } pH_i = 4.90 \times 10^{-4}$$

$$[HCl_i] = \frac{a}{H_i} = \frac{7.94 \times 10^{-6}}{4.79 \times 10^{-4}} = 16.2 \times 10^{-3}$$

$\gamma_{H_i} = .906$ and $\gamma_{Cl_i} = .878$ for the ionic strength of 16.2×10^{-3}

$$a_u = \gamma_{H_i}\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}\,a = .906 \times \frac{.911}{.878} \times 7.94 \times 10^{-6} = 7.46 \times 10^{-6}$$

$$C_v = \gamma_{H_i}\,C = .906 \times 2.57 = 2.33 \text{ gm./100 cc. } H_2O$$

Corrected $pH_i = 3.30$ (from curve for $C_v = 2.5$ and extrapolated for 2.33)

Corrected $H_i = 5.01 \times 10^{-4}$

$$\text{Corrected } [HCl_i] = \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} \times \frac{a}{H_i} = \frac{.911}{.878} \times \frac{7.94 \times 10^{-6}}{5.01 \times 10^{-4}} = 16.4 \times 10^{-3}$$

$$\text{Corrected } Cl_i = \gamma_{Cl_i}\,[HCl_i] = 14.4 \times 10^{-3}$$

$$Cl_0 = \gamma_{Cl_0}\,[HCl_0] = 7.4 \times 10^{-3}$$

$$\text{Total activity of ions inside} = H_i + Cl_i = 5.01 \times 10^{-4} + 14.4 \times 10^{-3} = 14.9 \times 10^{-3}$$

$$\text{Total activity of ions outside} = H_0 + Cl_0 = 9.8 \times 10^{-4} + 7.4 \times 10^{-3} = 8.4 \times 10^{-3}$$

$$\text{Donnan pressure} = 19.2 \times 10^3 \times (14.9 - 8.4) \times 10^{-3} = 125 \text{ mm. Hg}$$

This Donnan pressure when calculated by means of the pressure-elasticity equation is

$$D.P. = P + 8.1\,q - \frac{420}{q} = 3.5 + 151 - 22.5 = 132 \text{ mm. Hg}$$

which is identical, within the limits of error, with the value of 125 as calculated above.

Table III shows the values of the difference in Donnan pressure in mm. of Hg between inside and outside of the micellæ in 0.97 per cent gelatin solution of various pH as calculated by both methods. The same is shown on Fig. 8. These results confirm quantitatively the theory of viscosity of gelatin of various pH, as developed by Loeb, namely, that the viscosity is regulated by osmotic forces due to the Donnan equilibrium. A difference in the activities of the ions inside and outside of the micellæ is established because the concentration of the gelatin in the micellæ is greater than the outside concentration of the gelatin in the solution.

TABLE III.

The Donnan Osmotic Pressure in Micellæ of 0.97 Per Cent Gelatin-HCl Solution at 35°C.

Equivalent concentration of HCl	0	2.03×10^{-3}	4.06×10^{-3}	6.10×10^{-3}	8.13×10^{-3}	9.14×10^{-3}	11.2×10^{-3}	14.2×10^{-3}	20.3×10^{-3}	30.5×10^{-3}
pH_0	4.8	4.20	3.76	3.38	3.01	2.83	2.58	2.29	2.01	1.73
Relative viscosity	1.40	1.62	1.99	2.32	2.50	2.54	2.52	2.40	2.19	1.99
q	6.65	9.85	14.05	17.25	18.65	18.95	18.85	18.75	16.05	14.10
Concentration of gelatin in the micellæ $= \frac{.48}{q}$	7.22	4.87	3.41	2.78	2.57	2.53	2.54	2.56	2.99	3.40
H_0 from pH		6.3×10^{-5}	1.74×10^{-4}	4.17×10^{-4}	9.77×10^{-4}	1.48×10^{-3}	2.63×10^{-3}	5.13×10^{-3}	9.8×10^{-3}	18.6×10^{-3}
$Cl_0 = \gamma_{Cl_0}\,[HCl_0]$		1.9×10^{-3}	3.8×10^{-3}	5.6×10^{-3}	7.4×10^{-3}	8.3×10^{-3}	10.0×10^{-3}	12.6×10^{-3}	17.6×10^{-3}	25.6×10^{-3}
H_i		2.75×10^{-5}	8.5×10^{-5}	2.09×10^{-4}	5.01×10^{-4}	7.59×10^{-4}	1.48×10^{-3}	2.95×10^{-3}	5.98×10^{-3}	12.9×10^{-3}
$Cl_i = \gamma_{Cl_i}\,[HCl_i]$		4.4×10^{-3}	7.7×10^{-3}	11.2×10^{-3}	14.4×10^{-3}	16.1×10^{-3}	17.8×10^{-3}	21.9×10^{-3}	28.8×10^{-3}	36.9×10^{-3}
Donnan pressure mm.Hg $19.2 \times 10^3 \times (H_i + Cl_i - H_0 - Cl_0)$		42.0	73.0	104	125	134	128	138	138	107
Donnan pressure from relation $K\alpha/q - P + D.P. - E_1 q = 0$ $P = 3.5, K\alpha = 420, E_1 = 8.1$ mm.Hg		41.0	88.0	120	132	136	135	133	108	88

Effect of Concentration of Gelatin on the pH—Viscosity Curves.

With the increase in the total concentration of the gelatin solution the difference in the gelatin concentration between the micellæ and the outside is gradually diminished. Hence the increase in viscosity at pH 3.0 over that of isoelectric gelatin, which as shown above is brought about by the difference in the gelatin concentration of the micellæ, should become less conspicuous with increase in the total concentration of the gelatin solution. That this is exactly what happens is shown in Table IV.

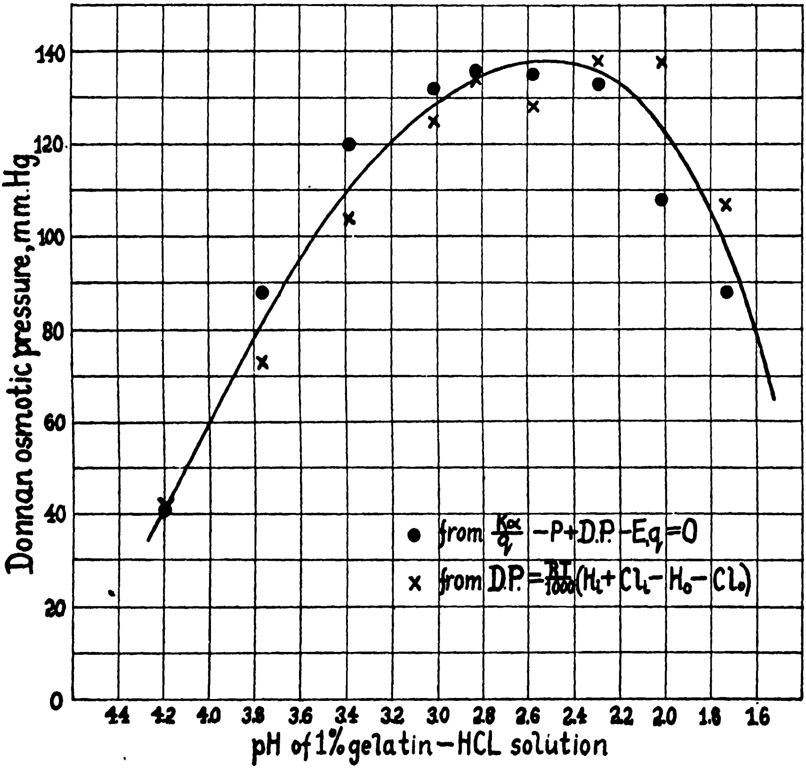

FIG. 8. The difference in the Donnan osmotic pressure between the micellæ and the outside solution at various values of pH in 1 per cent gelatin-HCl solution at 35°C. as calculated from viscosity measurements.

The effect of the concentration of the gelatin solution on the viscosity-pH curves is shown still more strikingly if instead of the relative viscosity values the values of q, *i.e.*, of the volume of H_2O taken up by a gm. of gelatin as calculated from equation (2), are used in plotting the curves. This is shown on Fig. 9. The enormous effect of the concentration of the gelatin on the viscosity that is caused by the Donnan equilibrium between the micellæ and the outside solution is apparent.

The curves show that at a concentration of 10 gm. of dry gelatin per 100 cc. of H_2O the Donnan effect on the viscosity of the solution disappears entirely. This indicates that at this concentration of gelatin there is no difference between the concentration of the gelatin inside and outside of the micellæ. Let α_1 be the fraction of each gm. of dry gelatin found in the micellæ, then the concentration of the gelatin inside of the micellæ is α_1/q since q is the volume of H_2O containing α_1 gm. of gelatin. Hence in a solution of gelatin containing 10 gm. of dry gelatin per 100 cc. of H_2O

TABLE IV.

Viscosity Measurement of Various Concentrations of Gelatin, pH 4.7 and pH 3.0, at 37°C.

Concentration in gm. per 100 cc. solution	0.5	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
Relative viscosity of gelatin, pH 4.7......	1.16	1.43	1.95	2.75	3.83	5.28	6.70	12.4	21.3
Additional viscosity = relative viscosity − 1..............................	0.16	0.43	0.95	1.75	2.83	4.28	5.70	11.4	20.3
Relative viscosity of gelatin, pH 3.0......	1.84	2.39	3.44	4.54	5.78	7.12	9.06	14.2	22.0
Additional viscosity.....................	0.84	1.39	2.44	3.54	4.78	6.12	8.06	13.2	21.0
Ratio of additional viscosity, pH 3.0/pH 4.7......................	5.24	3.23	2.57	2.02	1.69	1.43	1.42	1.16	1.03

$$\frac{\alpha_1}{q} = \frac{10}{100}$$

Substituting the value of q, we get

$$\frac{\alpha_1}{4.8} = \frac{10}{100}$$

or $$\alpha_1 = 0.48$$

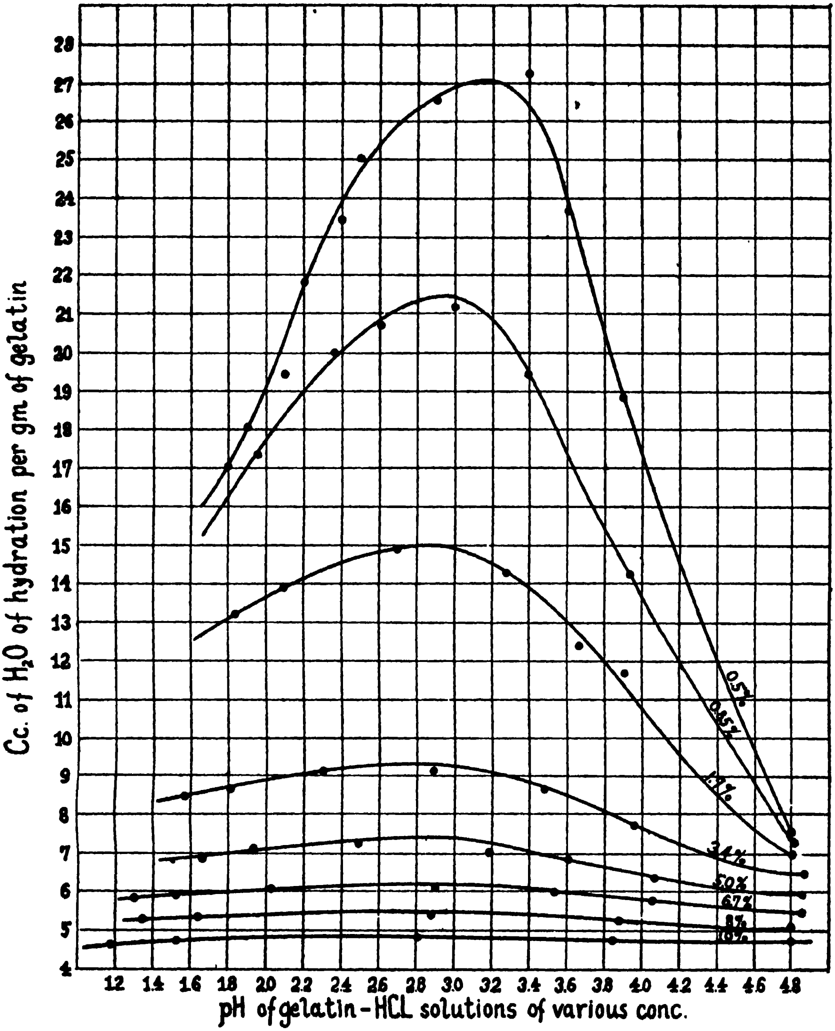

FIG. 9. Effect of concentration of gelatin on the pH-viscosity curves of gelatin-HCl solutions. The viscosity values are expressed in cc. of H_2O of hydration per gm. of gelatin.

This value of α_1 has been used in calculating the concentration of the gelatin in the micellæ of a 1 per cent solution of gelatin at various pH on the assumption that the relation between the fractions of the soluble and insoluble components of gelatin is not affected, within certain limits, by the total concentration of the gelatin or by the pH of the solution. The agreement between the values for the Donnan pressure in the micellæ as calculated by two independent methods shows that the assumption is correct.

SUMMARY.

1. It was shown that the high viscosity of gelatin solutions as well as the character of the osmotic pressure-concentration curves indicates that gelatin is hydrated even at temperatures as high as 50°C.

2. The degree of hydration of gelatin was determined by means of viscosity measurements through the application of the formula $\frac{\eta}{\eta_0} = \frac{1 + 0.5\,\omega}{(1 - \varphi)^4}$.

3. When the concentration of gelatin was corrected for the volume of water of hydration as obtained from the viscosity measurements, the relation between the osmotic pressure of various concentrations of gelatin and the corrected concentrations became linear, thus making it possible to determine the apparent molecular weight of gelatin through the application of van't Hoff's law. The molecular weight of gelatin at 35°C. proved to be 61,500.

4. A study was made of the mechanism of hydration of gelatin and it was shown that the experimental data agree with the theory that the hydration of gelatin is a pure osmotic pressure phenomenon brought about by the presence in gelatin of a number of insoluble micellæ containing a definite amount of a soluble ingredient of gelatin. As long as there is a difference in the osmotic pressure between the inside of the micellæ and the outside gelatin solution the micellæ swell until an equilibrium is established at which the osmotic pressure inside of the micellæ is balanced by the total osmotic pressure of the gelatin solution and by the elasticity pressure of the micellæ.

5. On addition of HCl to isoelectric gelatin the total activity of ions inside of the micellæ is greater than in the outside solution due to a greater concentration of protein in the micellæ. This brings about a further swelling of the micellæ until a Donnan equilibrium is established in the ion distribution accompanied by an equilibrium in the osmotic pressure. Through the application of the theory developed here it was possible actually to calculate the osmotic pressure difference

between the inside of the micellæ and the outside solution which was brought about by the difference in the ion distribution.

6. According to the same theory the effect of pH on viscosity of gelatin should diminish with increase in concentration of gelatin, since the difference in the concentration of the protein inside and outside of the micellæ also decreases. This was confirmed experimentally. At concentrations above 8 gm. per 100 gm. of H_2O there is very little difference in the viscosity of gelatin of various pH as compared with that of isoelectric gelatin.

The writer wishes to acknowledge his indebtedness to Dr. J. H. Northrop for valuable advice and suggestions.

STUDIES ON ENZYME ACTION.

XLV. Lipase Actions of the Whole Trout at Different Ages.

By K. GEORGE FALK, HELEN MILLER NOYES, and I. LORBERBLATT.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

(Accepted for publication, April 5, 1927.)

INTRODUCTION.

The lipase or ester-hydrolyzing actions of extracts of the whole rat at ages from 3 days before birth until 3 years 15 days, and of extracts of the whole mouse at ages from 6 days before birth until 1 year 261 days were presented in previous communications.[1,2] The changes in the "pictures" of relative actions on ten esters of these extracts as the animals increased in age were given in some detail, and the absolute enzyme actions, which were also recorded, were shown to undergo characteristic changes.

At the suggestion of Dr. C. B. Davenport of the Carnegie Institution of Washington, Station for Experimental Evolution, a similar study was undertaken with trout, because here it was possible to begin with the eggs at a very early stage, and to follow the enzyme actions to the adult fish. The materials were obtained from the State Fish Hatcheries, Cold Spring Harbor, Long Island, N. Y., and the investigation was made possible because of the constant cooperation and helpfulness of Mr. Stanley C. Walters, Superintendent of the Hatcheries.[3]

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[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925–27, viii, 75.

[2] Falk, K. G., and Noyes, H. M., *J. Gen. Physiol.* 1926–27, x, 359.

[3] Thanks are due the State Conservation Commission for permission to use the fish and eggs of the Hatcheries in this investigation, and Dr. C. B. Davenport for the use of laboratory space in the Station for Experimental Evolution where the greater part of the experimental work was done.

Experimental Methods.

Rainbow trout were used in this investigation. In studying the eggs, it was found that different results were obtained for the ester-hydrolyzing actions if whole eggs, finely ground, were used instead of aqueous extracts. It was therefore necessary to study the ground solid materials in a number of cases. In other respects, the experimental methods were essentially the same as those used in the earlier investigations.

The eggs were ground with a weighed amount of sea sand, then either extracted overnight with a definite quantity of water, or portions weighed out to which water was added and the ester-hydrolyzing actions determined directly. The very small fish were treated similarly. The larger fish were passed twice through a meat grinder and then either extracted with water or weighed portions used directly for the enzyme tests. The materials extracted with water were allowed to stand at room temperature overnight, filtered through paper, and portions of the cloudy or turbid filtrates used for the tests. For the experiments with the solids, the requisite amounts of water were added to the different portions and the enzyme tests made directly on these. Toluene was present throughout the extractions and the tests. The mixtures were brought to pH 7.0. The conditions of testing were the same as those described previously; 15 cc. of solution or mixture, 3.4 milli-equivalents of each of the ten esters, 22 hours incubation at 37°–38°, titration with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator; duplicate and blank determinations.

In a number of cases during the summer months, the eggs were removed from the fish after killing the latter. Most of the tests,

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----------- Original solids. ————Aqueous extracts.

FIG. 1. Relative lipase actions of trout eggs and whole trout, original solids and aqueous extracts.

Chart *a*. Eggs, 5 hours after being taken. Chart *b*. Eggs, 35 days after being fertilized. Chart *c*. Fish, 2 weeks after being hatched. Chart *d*. Fish, 2 weeks after being fed. Chart *e*. Fish, 3 months after being fed. Chart *f*. Fish, 4 to 5 years old.

The difference in the two curves (original solids and aqueous extracts) are quite marked in Chart *a*, less so in Chart *b*, and still less so in Chart *c*. The two curves practically coincide in each of the last three charts.

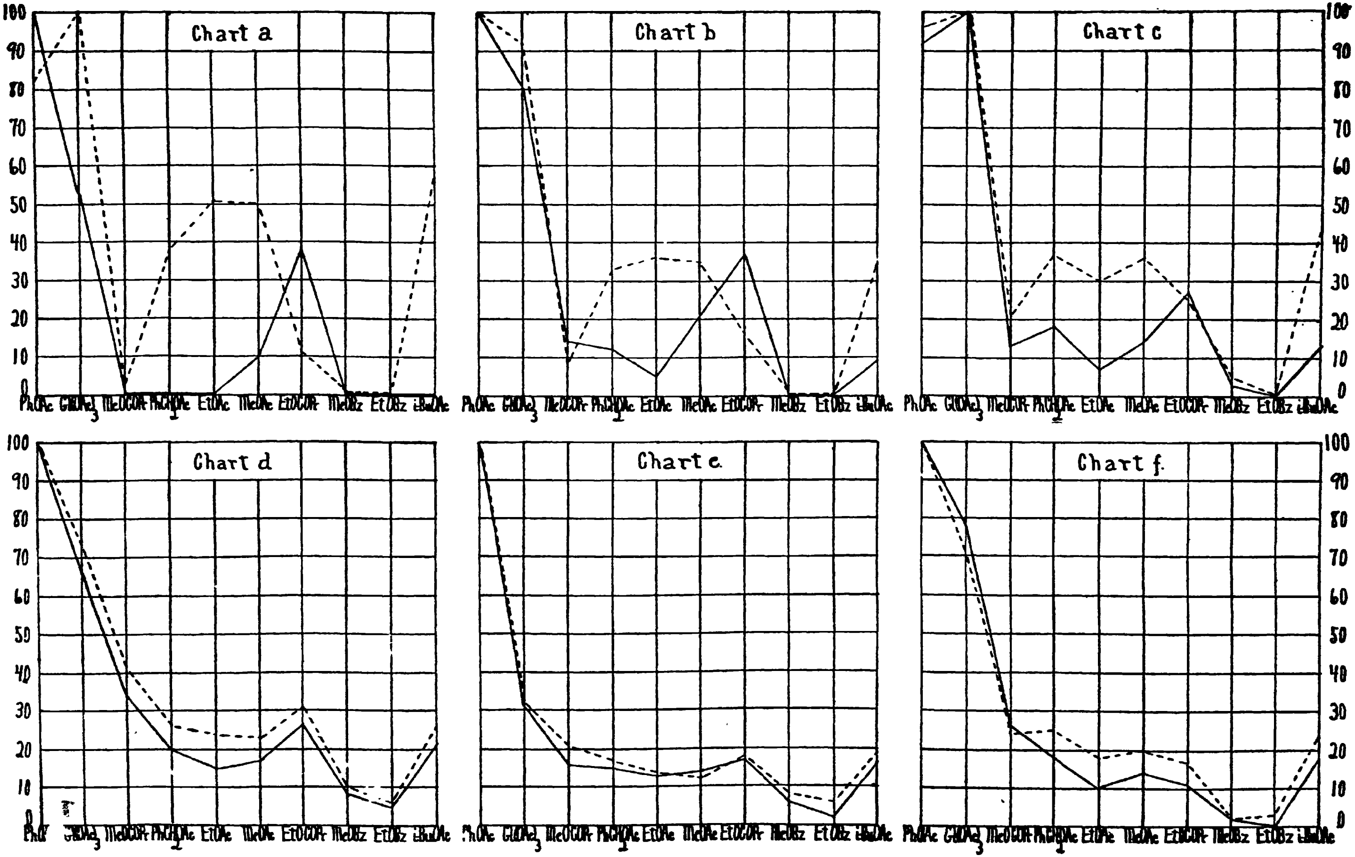
Chart a
Chart b
Chart c
Chart d
Chart e
Chart f
100
90
80
70
60
50
40
30
20
10
0
PhOAc
Gl(OAc)$_3$
MeOCOR
PhCH$_2$OAc
EtOAc
MeOAc
EtOCOR
MeOBz
EtOBz
iBuOAc

FIG. 1.

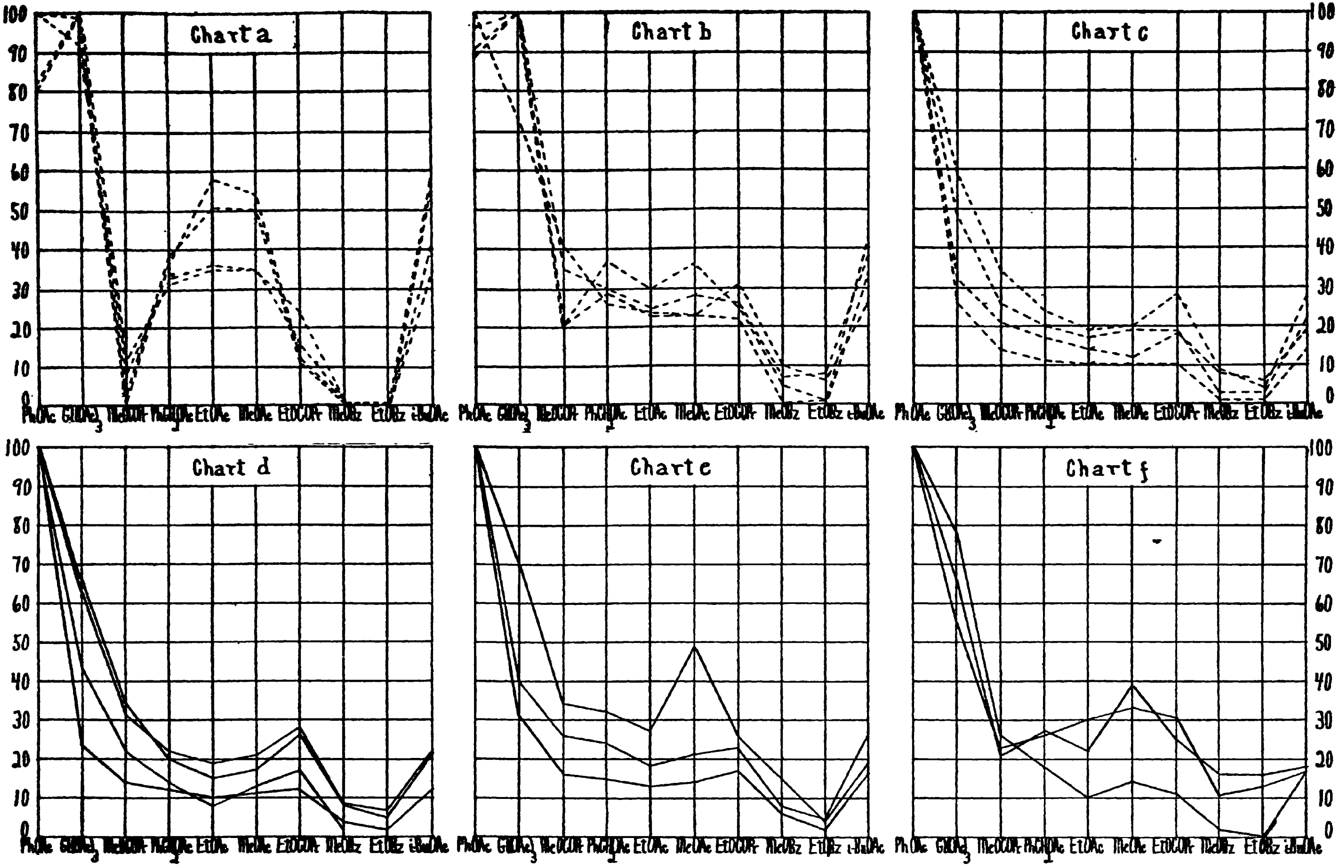
Chart a
Chart b
Chart c
Chart d
Chart e
Chart f
100
90
80
70
60
50
40
30
20
10
0
PhOAc
Gl(OAc)3
MeOCOPr
PhCH2OAc
EtOAc
MeOAc
EtOCOPr
MeOBz
EtOBz
i-BuOAc

FIG. 2.

however, were made with eggs taken for hatching purposes in November and later. These eggs were fertilized and kept in cold running water at the Hatchery in the usual way. They hatched after about 50 days, and began eating supplied food about 3 weeks after hatching. The ages of the older fish were only approximated.

EXPERIMENTAL RESULTS.

The relative actions on the ten esters of extracts and of original solids of the same materials are shown in Fig. 1. Six specimens at different stages of the life cycle were chosen, and each chart in the figure refers to one of these ages. It is evident that the "pictures" of the actions for the eggs at the earliest age chosen (Chart *a*) were entirely different for the extracts and the solids. The "pictures" differed considerably from each other at the next age (Chart *b*), but only to a small extent for the fish 2 weeks after hatching (Chart *c*). For the fish 2 weeks after feeding to the oldest studied, the curves were practically identical (Charts *d*, *e*, and *f*).

The eggs, for which the results are given in Fig. 1, were taken for hatching purposes. The absolute actions of the extracts of these eggs, some of which will be presented later, were comparatively small. The extracts of a number of sets of eggs removed after killing the fish, gave considerably larger absolute actions but essentially the same curves as the extracts of the more mature (but less soluble) eggs.

A considerable number of results was obtained with eggs and fish of different ages. In place of giving all of these results, a limited number are presented in Fig. 2. Since it is probable that where

----------- Original solids. ———— Aqueous extracts.

FIG. 2. Trout eggs and whole trout of different ages. Relative lipase actions, each curve representing the ester-hydrolyzing actions of the solid material (Charts *a*, *b*, and *c*) or of the aqueous extract (Charts *d*, *e*, and *f*).

The ages of the eggs and trout were as follows:

Chart *a*. Eggs, 5 hours after being taken to 5 days after being fertilized. Chart *b*. Fish, 2 weeks after being hatched to 2 weeks after being fed. Chart *c*. Fish, 3 weeks to 3 months after being fed. Chart *d*. Fish, 2 weeks to 2 months after being fed. Chart *e*. Fish, aged 3 months to 1½ years. Chart *f*. Fish, aged 2 years to 4 to 5 years.

The changes in the curves from the earliest eggs to the young fish are clear, but thereafter no regular change could be determined.

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Aqueous Extracts and Whole Solids of Trout Eggs and Whole Trout of Different Ages on the Indicated Esters.

Extracts	PhOAc	Gl(OAc)$_3$	MeOCOPr	PhCH$_2$OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	*i*-BuOAc
Eggs removed from fish	3.40	2.83	0.18	0.50	0.68	0.80	0.23	0.02	0.00	0.36
Eggs 4 days after fertilization	0.45	0.26	0.00	0.04	0.04	0.09	0.00	0.00	0.00	0.00
Eggs 37 days after fertilization	0.39	0.31	0.02	0.07	0.06	0.09	0.00	0.00	0.00	0.04
Fish, 2 weeks after hatching	0.72	0.78	0.10	0.14	0.05	0.11	0.21	0.02	0.00	0.10
Fish, 1 week after feeding	1.03	1.31	0.28	0.35	0.19	0.26	0.29	0.05	0.05	0.34
Fish, 2 weeks after feeding	3.14	2.08	1.08	0.64	0.48	0.54	0.84	0.26	0.16	0.68
Fish, 3 weeks after feeding	1.66	1.52	0.69	0.43	0.33	0.40	0.64	0.14	0.12	0.46
Fish, 3 weeks after feeding	3.48	1.16	0.66	0.62	0.60	0.62	0.60	—	—	0.56
Fish, 1 month after feeding	3.82	1.64	0.84	0.52	0.30	0.50	0.64	0.06	—	—
Fish, 2 months after feeding	3.62	0.84	0.52	0.45	0.36	0.40	0.43	0.14	0.06	0.44
Fish, 3 months after feeding	2.77	0.85	0.43	0.40	0.36	0.38	0.47	0.16	0.06	0.45
Fish, 6 months old	2.16	0.87	0.56	0.51	0.38	0.46	0.49	0.18	0.11	0.56
Fish, 1½ years old	0.73	0.51	0.26	0.25	0.18	0.45	0.24	0.13	0.05	0.14
Fish, 2 years old	1.21	0.43	0.25	0.30	0.21	0.29	—	0.10	0.14	0.21
Fish, 2½ years old	1.05	0.64	0.20	0.20	0.15	0.25	0.24	0.14	0.06	0.19
Fish, 4 to 5 years old	1.25	0.96	0.32	0.23	0.13	0.18	0.14	0.02	0.00	0.21
Solids										
Eggs, 5 hours after taking	1.66	2.02	0.06	0.76	1.03	1.00	0.23	0.00	0.00	1.17
Eggs, 15 days after fertilization	1.51	1.88	0.01	0.70	1.09	1.02	0.22	0.00	0.00	1.08
Eggs, 50 days after fertilization (just hatching)	2.09	2.07	0.26	0.66	0.74	0.74	0.50	0.00	0.00	0.85
Fish, 2 weeks after hatching	2.97	3.09	0.65	1.13	0.94	1.11	0.78	0.14	0.04	1.34
Fish, 3 weeks after feeding	3.38	2.48	1.39	0.88	0.81	0.77	1.06	0.33	0.20	0.89
Fish, 3 months after feeding	3.95	1.27	0.81	0.67	0.54	0.48	0.71	0.33	0.25	0.77
Fish, 4 to 5 years old	2.52	1.79	0.61	0.62	0.45	0.50	0.42	0.04	0.07	0.60

differences were found in the curves obtained with the solids and the extracts, the significant results are those obtained with the former, the results are shown for the eggs and the youngest fish in the first three charts for the ester-hydrolyzing actions of the solid materials, and in the last three charts for the extracts which are essentially the same as the results for the solids. Chart *c* for the solids and Chart *d* for the extracts refer to the same materials. The changes in the "pictures" of the actions of the youngest eggs up to the time that the young fish began to eat are quite clear. Thereafter the changes are not so distinct although some trends are discernible.

Absolute values of the ester-hydrolyzing actions of a number of the materials are shown in Table I. The results for extracts and whole solids are given for different ages of the eggs and fish. Not all the results which have been obtained are presented, but the data may be considered to be representative of the various actions. The concentrations in all cases refer to 44.4 mg. of original material per cc. of final solution or mixture tested.

Although the results presented in Table I are somewhat irregular, certain definite conclusions may be drawn from them. The extracts of the eggs removed from the fish after killing the latter (before October 1st) gave much larger actions than did the extracts of the eggs taken for hatching (after November 1st). Extracts of these more mature eggs gave only very small actions after fertilization as well as before. After hatching, the actions began to increase, markedly so 2 or 3 weeks after feeding, although some irregularities are apparent at this time, and apparently reached maximum values 1 to 2 months after feeding. For the older fish the actions decreased again.

With the whole solids, the absolute actions increased starting with the eggs attaining comparatively large values for the young fish.

Without going into the details of the absolute actions on all of the individual esters, certain facts of interest may be mentioned. With both extracts and solids, the actions of the eggs on methyl and ethyl butyrates were practically zero. Especially for the solids, the large actions on isobutyl acetate in comparison with the very small actions on ethyl butyrate (its isomer) are striking. After the fish had begun to feed, the actions on the butyrates increased markedly, to decrease again as the fish became older. In general terms, the acetic esters were hydrolyzed to considerable extents, although here there were considerable variations with the different alcohol radicals. The higher actions on glyceryl triacetate in comparison with those on phenyl acetate for the solids (and to a less extent for the extracts) with the eggs and youngest fish may be indicated. Other regularities might be pointed out but would not add anything of significance to the findings.

DISCUSSION.

In planning this investigation, it was hoped that the methods of enzyme study applied to the life cycles of whole rats and whole mice would be of interest with fish where it would be possible to procure eggs at a very early stage. The experimental study was complicated by the fact that with the eggs, solubility influences played a part, so that the "pictures" or curves of the relative ester-hydrolyzing actions were different for the extracts and whole solids. These differences did not exist with the fish after a short period of feeding. The changes in the "pictures" of enzyme actions from the eggs (solids) to the fish are quite clear, but there appeared to be no further changes of any degree of definiteness as the fish became larger and more mature. After the fish had eaten supplied food for several weeks the type of actions appeared to remain unchanged. Another fact which may have wider significance relates to the comparatively large solubility of the immature eggs in comparison with the insolubility (as far as substances carrying the ester-hydrolyzing actions are concerned) of the more mature eggs taken for purposes of hatching, although the types of the actions were the same for the two.

SUMMARY.

The ester-hydrolyzing or lipase actions of extracts and whole solids of trout eggs and whole trout of different ages were tested on ten simple esters by the method described in previous papers. Differences in solubility of the enzyme materials of the eggs were found. The "pictures" of the relative enzyme actions changed from a type found with immature eggs to a type which became constant for the fish after they had eaten for 2 weeks. After this, the type did not change up to the age of 4 to 5 years (the oldest trout studied). The absolute ester-hydrolyzing actions of the materials were also presented and discussed.

STUDIES ON ENZYME ACTION.

XLVI. The Ester-Hydrolyzing Actions of Whole Trout Preparations under Various Conditions.

By HELEN MILLER NOYES, I. LORBERBLATT, and K. GEORGE FALK.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

(Accepted for publication, April 5, 1927.)

INTRODUCTION.

The ester-hydrolyzing actions of whole trout and trout egg preparations under the conditions of the general investigation in progress in this laboratory offer a comparatively new field of study so that it seemed of interest to determine some of the simpler questions connected with these enzyme actions. In an earlier paper[1] the actions at different temperatures were studied because it was considered possible that the low temperature at which the fish lived might influence the actions of enzymes under different temperature conditions. It was found, however, that the general behavior was no different from that of castor beans, for example, for which the conditions of growth required a comparatively high temperature. In general terms, two main opposing actions were involved in studying the enzyme actions at different temperatures; increase in enzyme action due to rise in temperature, and inactivation of enzyme by heat in the course of the experimental tests. These two factors did not produce the same effects with the actions on the different esters, so that, depending upon the length of time of the action, greater hydrolysis was found at lower temperatures with some of the esters.

In the preceding paper,[2] the ester-hydrolyzing actions of extracts and whole solids of trout eggs and whole trout at different ages were presented, and the changes in the curves of relative actions and the absolute actions considered.

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[1] Noyes, H. M., Lorberblatt, I., and Falk, K. G., *J. Biol. Chem.*, 1926, lxviii, 135.

[2] Falk, K. G., Noyes, H. M., and Lorberblatt, I., *J. Gen. Physiol.*, 1926–27, x, 837.

In this paper,[3] some of the results obtained in the study of the ester-hydrolyzing actions at different hydrogen ion concentrations will be described as well as the actions of extracts obtained with different solvents.

Experimental Methods.

The experimental methods were essentially the same as those described and used in the preceding paper. Adult fish only were used. These were killed, passed twice through a meat chopper, solid portions weighed directly when desired or treated with a definite volume of solvent and toluene added. After extracting overnight the mixtures were filtered through paper, and treated in various ways. For the experiments at different hydrogen ion concentrations, sodium hydroxide or hydrochloric acid was added and the mixtures tested with indicators. For dialysis collodion bags were used. The mixtures or extracts were finally diluted to the proper concentrations, and 15 cc. portions used for the tests on 3.4 milli-equivalents of each of the ten esters in the usual way. Incubation was allowed to proceed for 22 hours at 37°–38° or at 15°–17°, the acid formed being determined with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator. Toluene was present throughout, and duplicates and the usual blanks run.

EXPERIMENTAL RESULTS.

Some results bearing on the ester-hydrolyzing actions at different initial hydrogen ion concentrations are presented in Table I. Experiment F 2 shows that at both temperatures of testing, higher actions were obtained at pH 7.0 than in the more acid solutions. The fact which was brought out in the earlier paper, namely, that some of the actions, especially the action on benzyl acetate, are larger at

[3] The rainbow trout used were obtained through the kindness and courtesy of Mr. Stanley C. Walters of the State-Fish Hatchery, Cold Spring Harbor, Long Island, N. Y., by permission of the State Conservation Commission. The writers wish to thank the Commission and Mr. Walters for the aid they have given in making possible the carrying out of this investigation.

the lower temperature if the tests are continued over a sufficient period of time (22 hours in the present instance), is also shown here. The comparatively large concentration of this extract showed the actions at the different hydrogen ion concentrations more clearly than did the results of Experiment F 8 with a more dilute extract. The actions in this experiment were clearly larger at pH 7.0 and 8.0 than at pH 6.0, but there were no clear-cut differences between the actions at pH 7.0 and 8.0.

In view of the extended study made of the lipolytic actions of various tissue and tumor extracts at different hydrogen ion concentrations published some years ago,[4] it appeared to be unnecessary to go farther in this direction with the trout extracts, as the results paralleled the earlier findings. It was there pointed out that in the determination of enzyme action at a definite hydrogen ion concentration, two factors operate simultaneously in every case; enzyme action at the given hydrogen ion concentration, and inactivation of enzyme at that hydrogen ion concentration. Also, in the study of lipase actions, the hydrogen ion concentrations of the mixtures may change because of the formation of acid as the action proceeds.

TABLE I.

Ester-Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Aqueous Trout Extracts in 22 Hours.

	Experiment F 2. Tested 15–17.5° 88.9 mg. per cc.			Experiment F 2. Tested 37.5° 88.9 mg. per cc.			Experiment F 8. Tested 37.5° 44.4 mg. per cc.		
pH	5.0	6.0	7.0	5.0	6.0	7.0	6.0	7.0	8.0
PhOAc	0.75	1.59	2.15	0.87	1.71	2.33	0.54	0.66	0.61
Gl(OAc)$_3$	0.22	0.74	1.20	0.50	0.95	1.22	0.26	0.47	0.59
MeOCOPr	0.15	0.53	0.63	0.39	0.47	0.43	0.21	0.15	0.26
PhCH$_2$OAc	0.17	0.59	1.03	0.36	0.46	0.57	0.19	0.23	0.23
EtOAc	0.08	0.14	0.35	0.17	0.19	0.37	0.09	0.19	0.32
MeOAc	0.00	0.26	0.54	0.18	0.26	0.51	0.06	0.35	0.46
EtOCOPr	0.03	0.16	0.29	0.30	0.30	0.57	0.08	0.17	0.20
MeOBz	0.04	0.08	0.12	0.27	0.10	0.39	0.12	0.12	0.22
EtOBz	0.03	0.04	0.02	0.23	0.07	0.22	0.06	0.17	0.12
i-BuOAc	0.03	0.43	0.71	0.29	0.30	0.51	0.05	0.11	0.21

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[4] Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Biol. Chem.*, 1923, lv, 653.

TABLE II.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Various Trout Preparations on the Indicated Ester in 22 Hours.

No. of experiment	Mixture tested	Temperature of tests	PhOAc	Gl(OAc)$_3$	MeOCOPr	PhCH$_2$OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	*i*-BuOAc
F 9	Water extract	37½°	0.57	0.29	0.17	0.16	0.17	0.16	0.14	0.18	0.01	0.09
	Glycerol extract, *5 per cent*	"	0.61	0.28	0.19	0.20	0.18	0.13	0.15	0.15	0.11	0.15
	" " *20* " "	"	0.91	0.48	0.29	0.23	0.13	0.15	0.17	0.12	0.01	0.22
	" " *50* " "	"	1.01	0.46	0.30	0.28	0.18	0.19	0.23	0.07	0.06	0.16
F 12	Water extract	37½°	0.73	0.51	0.26	0.25	0.18	0.45	0.24	0.13	0.05	0.14
	NaCl " *10 per cent*	"	0.75	0.61	0.13	0.27	0.15	0.25	0.17	0.09	0.25	0.17
	Glycerol extract, *50 per cent*	"	0.92	0.49	0.34	0.22	0.17	0.26	0.18	0.06	0.09	0.20
F 21	Water extract	15°–17°	1.37	0.63	0.43	0.61	0.20	0.27	0.22	0.14	0.10	0.41
	" " (dialyzed vs. running water)	"	0.58	0.18	0.14	0.14	0.08	0.09	0.11	0.07	0.01	0.14
	NaCl " *10 per cent*	"	1.33	1.16	0.74	0.99	0.72	1.02	0.47	0.15	0.11	1.02
	Glycerol extract, *50 per cent*	"	1.47	0.82	0.61	0.58	0.37	0.48	0.37	0.08	0.02	0.47
	" " *50* " " dialyzed vs. distilled water; liquid in bag	"	1.14	0.53	0.41	0.30	0.17	0.23	0.19	0.04	0.01	0.26
	Glycerol extract *50 per cent*, dialyzed vs. distilled water; (outside liquid no action) liquid in bag + outside liquid	"	1.34	0.68	0.51	0.46	0.26	0.38	0.28	0.07	0.03	0.44
	Original solid	"	2.23	1.74	1.08	1.25	0.76	0.86	0.68	0.29	0.19	1.40
	Solid, after dialysis vs. distilled water	"	1.01	0.72	0.26	0.38	0.31	0.27	0.23	0.00	0.00	0.42
	Liquid in bag from solid after dialysis vs. distilled water	"	1.27	0.68	0.40	0.44	0.34	0.31	0.26	0.10	0.06	0.44
	Solid after dialysis vs. distilled water + liquid in bag	"	1.90	1.34	0.80	0.82	0.60	0.68	0.74	0.12	0.10	0.98
	" " " " " " (outside liquid no action alone) + outside liquid	"	1.17	1.01	0.45	0.55	0.50	0.45	0.27	0.10	0.00	0.68

	Outside liquid + liquid in bag from solid dialyzed vs. distilled water	"	1.34	0.84	0.47	0.57	0.32	0.42	0.24	0.05	0.00	0.50
	Solid after dialysis vs. distilled water + liquid in bag + outside liquid	"	2.12	1.51	0.90	0.97	0.64	0.80	0.65	0.13	0.10	1.09
F 22	Original solid	14°	1.61	1.33	0.53	0.63	0.43	0.60	0.37	0.07	0.06	0.60
	Water extract	"	0.86	0.60	0.25	0.27	0.16	0.25	0.14	0.04	0.02	0.28
	Glycerol extract, *50 per cent*	"	0.88	0.74	0.34	0.30	0.21	0.28	0.22	0.03	0.00	0.27
	NaCl " *10* " "	"	0.99	0.91	0.36	0.44	0.39	0.45	0.29	0.05	0.05	0.47
	" " *10* " " dialyzed vs. distilled water, liquid in bag	"	0.76	0.71	0.28	0.21	0.16	0.21	0.15	0.00	0.00	0.22
	NaCl extract, *10 per cent*, dialyzed vs. distilled water (outside liquid no action alone) liquid in bag + outside liquid	"	1.06	0.93	0.36	0.42	0.33	0.43	0.25	0.02	0.00	0.41

"The difficulty of an exact interpretation of the most favorable hydrogen ion concentration for such lipase actions is therefore apparent. The study of the actions at different hydrogen ion concentrations, definite at the commencement of the actions, involves changes in the reactions and therefore gives results under changed, or continuously changing, conditions. The study of the rates of inactivation by testing at the same hydrogen ion concentration, after permitting the mixtures to stand at different hydrogen ion concentrations, yields definite conclusions, but really involves a problem essentially different from that of actions at definite hydrogen ion concentrations. . . . . It is, of course, possible that the comparative rates of action and of inactivation of certain lipase preparations may be such that definite optima are obtained at certain hydrogen ion concentrations. In the experiments described here,[4] it is seen that the greater actions in the more alkaline solutions more than make up for the inactivations in these solutions, while in the more acid solutions, the reverse is the case."

In view of the results with trout extracts and the general conclusions just stated, it may suffice to state that favorable conditions for testing the trout extracts would be in the neighborhood of pH 7.0, that the meaning of optimum hydrogen ion concentration for the actions must be more sharply defined to have a real significance and may even then serve no practical purpose. In general terms, similar relations apply to the work with trout as with the other tissues and tumors studied. Further results will therefore not be communicated in this connection.

A large number of experiments was carried out in connection with various solvents for extraction and the behaviors of the extracts on dialysis, etc. The four experiments for which the results are given in Table II may be looked upon as representative of the general findings.

The trout for which results are given in Table II were 1½ to 2½ years old. The experiments were carried on at pH 6.0 for F 9, and at pH 7.0 for the rest. The concentrations for the enzyme materials for these experiments corresponded to 44.4 mg. of original material per cc. of solution tested. In the preparation of the extracts, amounts of ground fish were weighed out and liquid added to correspond to three times this concentration. This permitted increases in volume in dialysis and still allowed the final concentrations to be the same. In every case, therefore, before the enzyme tests were carried out, the extracts were made up to three times their original volumes with water. This meant that where salt or glycerine solutions were used for the extractions, their concentrations were reduced to one-third in the enzyme tests. Thus, where the extract was stated to be made with a 50 per cent glycerol solution, the enzyme tests were carried out with a 16⅔ per cent glycerol solution, etc.

The results of the four experiments as far as use of different solvents was concerned, showed that glycerol solutions, especially the higher concentrations, extracted more of the active enzyme material than did water alone. The differences, while not large, were still appreciable. There were no such differences with the sodium chloride solution extracts, the enzyme activities, at times slightly larger, were in general much the same as for the aqueous extracts. The results for the more dilute sodium chloride solutions, while not given, were essentially the same as for the 10 per cent solutions. To sum up, it may be said that the glycerol and sodium chloride solutions extracted at least as much of the active enzyme material from the fish as did the water, and in some cases extracted more.

In the dialysis experiments, it was found possible to separate out material, inactive in itself, while the material from which it was separated showed decreased activity. Combining the two, the original activity was restored. In other words, a so called coenzyme was shown to exist, analogous to the results with eel preparations.[5] In the present connection this was shown to be present in Experiment F 21 with the 50 per cent glycerol extract, in Experiment F 22 with the 10 per cent NaCl extract. There were indications of the same relations with the solid material of Experiment F 21 for some of the ester actions, but the differences were not sufficiently large or uniform to warrant definite conclusions.

SUMMARY.

The ester-hydrolyzing actions of trout extracts at different hydrogen ion concentrations were studied. It was pointed out that the behavior was similar to that found with various tissue and tumor materials and that similar relations hold.

Glycerol solutions extracted somewhat larger amounts of active enzyme material from trout than did water and sodium chloride solutions, although the differences were not large. Evidence was presented for the existence of a so called "coenzyme."

[5] Noyes, H. M., Lorberblatt, I., and Falk, K. G., *J. Gen. Physiol.*, 1926–27, x, 1.

THE ANALYSIS OF THE DIVISION RATES OF CILIATES.

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(Accepted for publication, April 11, 1927.)

The existence of data on the division rates of representatives of three orders of ciliate protozoans grown in pedigree isolation culture with the same culture medium for 3 years (Dawson)[1] invites analysis to determine the influences which affect the division rates and the relations between the respective division rates.

The division rates of *Paramecium aurelia* (mutant), *Blepharisma undulans*, and *Histrio complanatus* were determined by Dawson. The fact that the culture conditions were kept the same for all three organisms, at any given time, insures homogeneous data.

I.

The changes in the division rate of unicellular organisms are probably best indicated by a staggered or running average; a convenient time interval is 10 days. Averages of the 10 day daily rates made at the end of 10 day periods show the same changes, but with less objective clearness and accuracy. Running averages and the corresponding 10 day averages, for a few days, are plotted in Fig. 1 to show the relations between them. The histogram plot, commonly used by protozoologists, implies that the average rate was maintained constant for the entire 10 day period. Consequently, the histogram is not only theoretically incorrect but may be misleading practically in the interpretation of data.

The data analyzed in this paper are those of 10 day division rates per animal, per day, for each species, for the period between January 30, 1924, and January 14, 1927; with the exception that the *Paramecia* rates started on July 18, 1924, and that the *Histrio* line died on Feb. 8, 1926.[1] The rates for these organisms are separately plotted in Fig. 2, *a*, *b*, *c*.

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[1] These observations have been published in part, and the rest will be published later. Dawson, J. A., 1926, *J. Exp. Zool.* xliv, 133; 1926–27, xlvi, 345.

II.

Inspection of the curves representing the division rates for these organisms shows that there are distinct secular trends for each curve and that each trend has a different slope. It is therefore necessary to remove these trends before we can compare the division rates. The ordinates of a line of trend are determined for the beginning, center, and the end of the curve and the line is drawn through these ordinates of trend.[2] The resulting trend is then removed graphically with the aid of dividers. Two sources of error are possible with this method. One is an error made in measurement with the dividers. Since the scale of the original graphs was chosen so that the distance between units was 5 mm., an error of this magnitude is not probable with care-

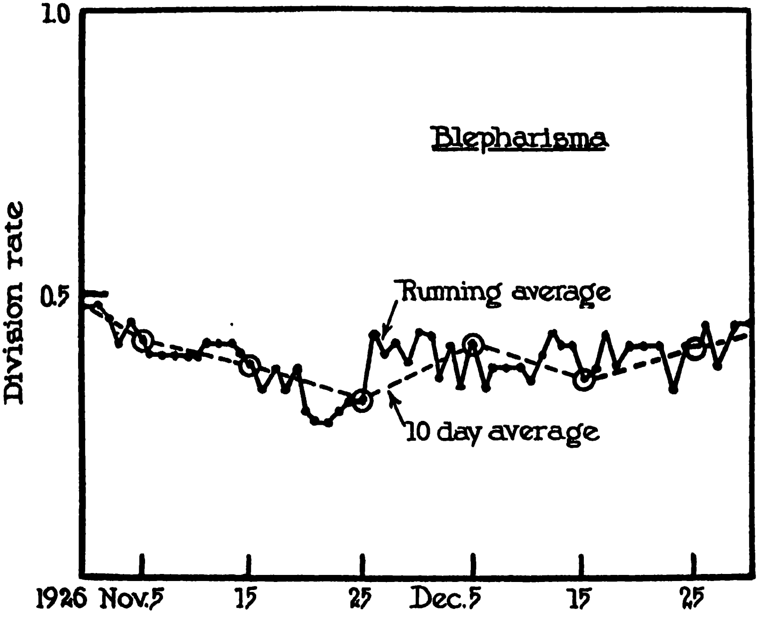

FIG. 1. The comparison between 10 day and running averages.

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[2] Rietz, H. L., Handbook of mathematical statistics, New York, 1924, 159.

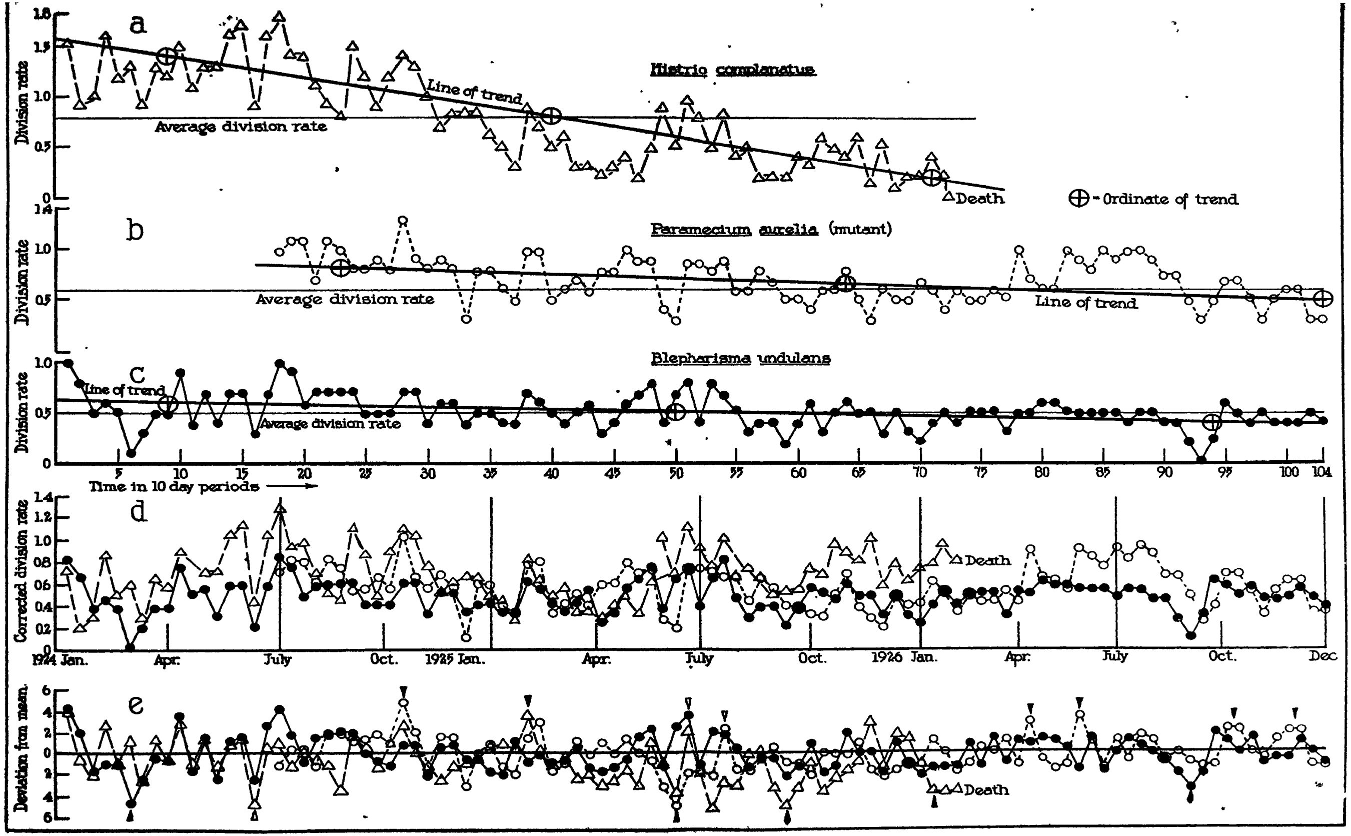

FIG. 2. *a.* Average division rates and line of secular trend of *Histrio complanatus*. *b.* Average division rates and line of secular trend of *Paramecium aurelia*. *c.* Average division rates and line of secular trend of *Blepharisma undulans*. *d.* Average division rates corrected for secular trend. *e.* Division rate residues after minimizing secular trend and seasonal variation. △ *Histrio complanatus*. ○ *Paramecium aurelia*. ● *Blepharisma undulans*. ▼ Deviations associated with a change in water source. ▲ Same as ▼. ▽ Deviations associated with removal to Woods Hole or to Cambridge. ◆ Deviations associated with temporary change of technician during the absence of Dawson.

ful measurement. The other error is due to the fact that the correction should be made proportional to the magnitude of the trend rather than to the actual departure from the ordinate of trend. This error is greater with steeper slopes of the trend line. The correction is negligible for the *Paramecia* and *Blepharisma* data and calculation showed that the *Histrio* correction would not be sufficiently improved to justify the greater effort of the numerical over the graphical correction.

III.

The curves corrected by the removal of the effect of secular trend are plotted together in Fig. 2, *d*. The division rates now show a cyclic rhythm with a period of 1 year in length, and with the maximum of the cycle occurring during the summer. The first cycle is most distinct, the two following ones showing less effect of season on the division rates.

By suitable means[3] the extent of this seasonal cycle was determined, and the cycle for each organism is plotted in Fig. 3. The effect of the seasonal variation was then removed from the curves previously corrected for secular trend, and the residues are shown in Fig. 2, *e*. In order that the comparative variations may be clearly indicated the means for the division rates are superimposed on this graph.

IV.

These residual curves may now be directly compared since the disturbing effects of seasonal variation and of secular trend have been minimized. The maximum correlation of the corrected *Paramecium* rates (x) with the *Blepharisma* rates (y) is their partial correlation independent of time,[4] and is $r_{xy \cdot t} = -0.08$. The *Histrio* rates (z) may be correlated with the composite of the *Paramecium* and *Blepharisma* rates by means of the multiple correlation[5] $r_{z \cdot xy} = 0.29$. These correlation coefficients indicate no relation between the corrected division rates. The distribution of these residual rates about their means suggests a chance distribution.

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[3] Rietz (1924),[2] 151–159. The seasonal variation is minimized by means of the composite seasonal effect, Fig. 3. Sufficient data are not available for complete correction for the decreasing seasonal effect.

[4] Rietz (1924),[2] 164.

[5] Rietz (1924),[2] 145.

V.

If all conditions influencing the division of the protozoans could be kept constant we should expect the organisms to divide regularly and the probability of occurrence of division would be constant. By means of the Lexian ratio we may test the "corrected division rate data" to see if the divisions occur with a constant probability. The empirical probability of a division may be calculated from the average division rate and the sum of the divisions. From this information it is possible to determine the numerical value for the relative Bernoulli standard deviation for our series; then the Lexian ratio is the ratio of the relative standard deviation of the series to the relative Bernoulli standard deviation for the same series.

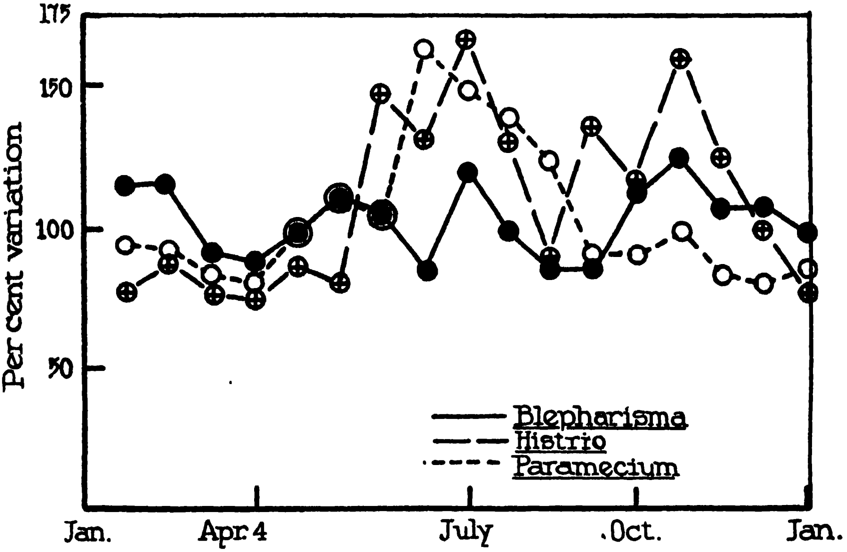

FIG. 3. The yearly cycle of seasonal variation for each organism.

The numerical values for the Lexian ratio are given for each organism in Table I for the original data, for the corrected data, and for the corrected data omitting those observations known to be caused either by a change of medium, or by removal from Cambridge to Woods Hole, or return, or by changes in culture technique.

Purely chance deviations of a constant probability would give a Lexian ratio close to unity. The deviations between the calculated ratios and unity are probably due to variations in technique and in culture conditions that we cannot completely describe at present.

VI.

The analysis shows that after the effect of long secular trend is removed the influence which causes similar variations in the division rates of these three ciliates of diverse orders is a seasonal one. The period of this rhythm is yearly and the maximum of the effect occurs in July. The effect of this rhythm remains distinct although it is disturbed by the annual moves to and from Woods Hole at this time. The effect of the rhythm becomes less the longer the organism is maintained under laboratory conditions. The 1st years of Woodruff's 8 year culture[6] and of Metalnikow's 10 year culture[7] show seasonal rhythms that diminish under laboratory conditions. The mechanism of this diminished seasonal effect could be determined under known conditions, and it would be of interest to do so.

TABLE I.

The Lexian Ratios of the Division Rate Residues after Correction for Secular Trend and Seasonal Variation.

Organism	Original data	Corrected data	Corrected data less known disturbances
Paramecium aurelia	2.19	1.54	1.19
Blepharisma undulans	1.81	1.61	1.31
Histrio complanatus	3.28	1.50	1.21

These same series also show definite secular trends. The trend in Woodruff's *Paramecium aurelia* is upward and distinct. That of Metalnikow's *Paramecium caudatum* is also upward but with less slope. This analysis of our own division data leaves no trace of cycles or rhythms that might be attributed to recurring cellular reorganization.[8] The four "cycles" from Calkins that Wilson[9] publishes may be explained by noting that the first two are due to a change to beef extract culture medium; and that the third and fourth, after they are corrected for the steep trend, show one yearly, seasonal cycle very similar to those of our own data and to those of the 1st years of Woodruff's and of Metalnikow's curves.

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[6] Woodruff, L. L., 1921, *Proc. Nat. Acad. Sc.*, vii, 41.

[7] Metalnikow, S., 1922, *Compt. rend. Acad.*, clxxv, 776.

[8] A forthcoming paper by Dawson will contain a further discussion of this problem.

[9] Wilson, E. B., The cell in development and inheritance, New York, 3rd edition, 1925, 241.

VII.

SUMMARY.

1. Analysis of the division rates of *Paramecium aurelia* (mutant), *Blepharisma undulans*, and *Histrio complanatus* grown separately in pedigree isolation culture with the same culture medium, and in the same room at any given time, for a period of 3 years, discloses a secular trend and a seasonal rhythm for each organism. The seasonal rhythm is a yearly cycle with a maximum during July.

2. After removal of the effects of trend and seasonal rhythm, no correlation is found between the division rates of the several organisms. The distribution of the division rates is then one of chance order, except for large deviations known to be associated with changes in the culture technique. Each organism has a division rate varying independently of the others.

3. Consequently, seasonal rhythm alone has forced similar variations in the division rates of these three protozoans. The seasonal effect is gradually lost when the animals are raised for several years under laboratory conditions Examination of the literature discloses other similar cases.

4. It is clear that unless all of the conditions of experiment are kept *constant,* one must analyze all protozoan division rate data in some such manner as that here presented before any conclusions may be drawn as to the existence of "cycles" or "rhythms."

STUDIES ON THE AMOUNT OF LIGHT EMITTED BY MIXTURES OF CYPRIDINA LUCIFERIN AND LUCIFERASE.

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(Accepted for publication, May 3, 1927.)

INTRODUCTION.

Interest has centered for a long time on the intensity and efficiency of animal luminescence, not only from a theoretical but also from a practical standpoint. With the exception of the beetles, most luminous animals emit light in such small amounts that a dark-adapted eye is required to register their intensities. It is a difficult matter to measure accurately low light intensities, especially when they are intermittent as in certain luminous organisms. The intensities of *Cypridina*, however, are sufficiently bright to measure.

Harvey, 1925, has successfully measured the amount of light and over-all luminous efficiency of dense emulsions of luminous bacteria, and finds that they are 0.156 per cent efficient. As is well known the light production in *Cypridina* is an oxidation process, due to the oxidation of a substrate, luciferin, in the presence of a catalyst, luciferase, and follows the laws of enzyme action. After mixing luciferin and luciferase solutions a gradual decrease of luminous intensity follows the initial marked brilliancy. Studies of these decay curves by Amberson by a photographic method show that the light intensities are continually and gradually falling off. In general the effect of the enzyme, luciferase, is proportional to its concentration. Amberson's curves show that the reaction velocities follow this law, *i e.* the velocity constant is proportional to the concentration of the enzyme. Amberson assumed after Trautz that, ". . . if light intensity at any instant is assumed to be proportional to reaction velocity at that instant, the decay curve of the light produced in the course of the luminescent reaction in *Cypridina* is, after the first second, in com-

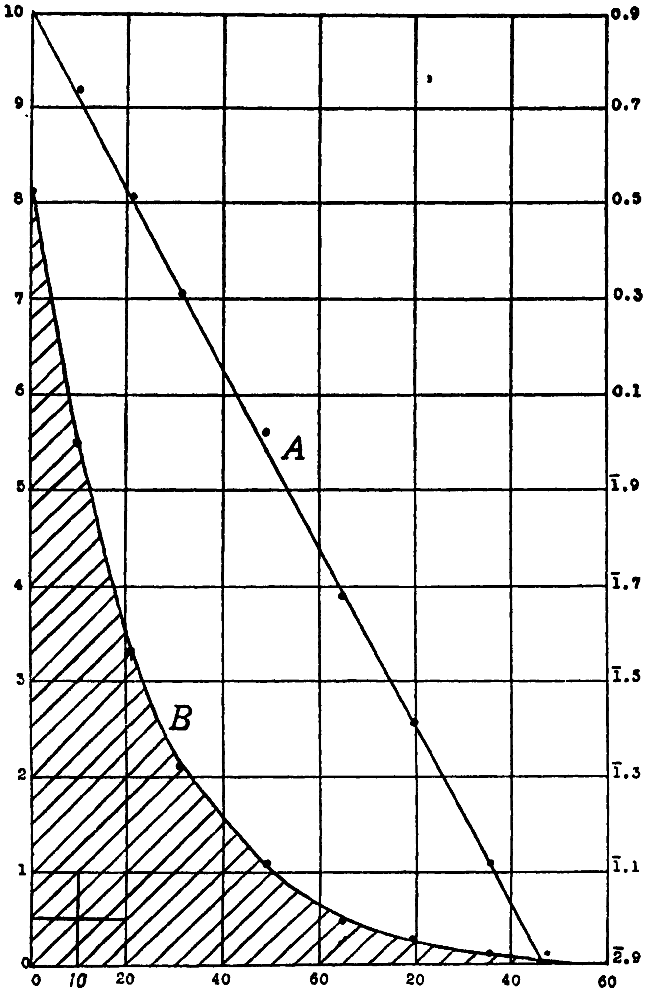

FIG. 1. Typical curve of *Cypridina* luminescence. *A*, logarithmic plotting; *B*, ordinary plotting of luminescence intensity against time. Ordinates, right, logarithms of intensity; ordinates, left, intensity in arbitrary units. Abscissæ represent time in seconds.

plete agreement with the theoretical expectation for a monomolecular reaction." In other words the rate of change of the concentration of *A* at any instant is proportional to its concentration at that instant, or

$$dx/dt\alpha(a-x)$$

where a is the number of molecules of A present originally, and x is the number of molecules of A changed in t minutes; $(a-x)$ is the concentration of A after t minutes or

$$dx/dt = k_1 (a-x)$$

where k_1 is the velocity constant. k_1 has a characteristic value for such reactions and is a measure of the rate of reaction. By integration one obtains

$$k = 1/t \ ln \frac{a}{a - x}.$$

When the intensities are plotted against time, a typical decay curve is obtained (Fig. 1), and when the logarithms of intensities are plotted against time, a straight line is obtained. Since the reaction velocities are proportional to the enzyme concentration, the velocity constants should vary with the luciferase concentrations, but not with the luciferin concentrations. Logarithmic plottings of different luciferin concentrations are parallel, provided the luciferase is present in the same concentrations, whereas the logarithmic plottings of different luciferase concentrations have different slopes.

The present paper records experiments carried out with *Cypridina* luciferin and luciferase in an attempt to measure the total amount of light emitted with various concentrations of the substrate and enzyme. The photographic method of Amberson was adequate for measuring the intensities in arbitrary units and the reaction velocities, but is unadaptable for determining the total amount of light emitted by a unit volume of luminous material in lumens.

A problem concerned with the dynamics of bioluminescence is beset with the difficulty of dealing with a very complex system. All attempts to purify either the enzyme or the substrate are unsuccessful. Furthermore, to obtain the luminous substances, the whole dried body of the organism must be extracted. Hence one is trying to measure one reaction, resulting in luminous intensity, in a system which may exhibit secondary oxidations, or additional modifying factors. In the following experiments anomalous and inexplicable factors control certain features of the reaction.

Materials and Methods.

Cypridina hilgendorfii Müller, the Japanese ostracod, was the organism which was used for the source of light in all the experiments. The desiccated animals were powdered and kept over $CaCl_2$. Great variations occur in the brilliancy of the luminescence from such powder depending upon the care with which the animals were originally gathered and dried, as well as the methods employed in preparing the extracts. It is necessary, therefore, to have the best available stock for studies upon luminescence.

The aqueous solutions of both the enzyme, luciferase, and the substrate, luciferin, have been freshly prepared for each series of experiments. In this way the minimum deterioration of solutions has been obtained. In no case has the enzyme been used after 6 hours from the time of preparation. Luciferase has been prepared in the standard manner by extracting the dried powder with cold sea water, or ordinary distilled water, and allowing the solution to stand until the contained luciferin has been completely oxidized to oxyluciferin. Such a solution is entirely non-luminous to a dark-adapted eye. The solution is then filtered and kept as the stock enzyme solution. Three series of experiments were completed with a distilled water extract; in all others sea water extracts were used. In all cases 0.25 gm. of powder to 50 cc. water have been used in preparing the luciferase.

The luciferin solutions were prepared in two different ways. Ordinarily 1 gm. of dried powder was extracted with 100 cc. N/10 HCl acid sea water. The *Cypridina* powder was added to the sea water at the moment of boiling and the boiling continued for a few moments in order to completely destroy all the enzyme. Cooling to room temperature immediately followed by allowing the flask to stand undisturbed in a vessel of cold water. During the cooling process the insoluble particles of powder settle to the bottom of the flask leaving a relatively clear yellowish solution of luciferin. The supernatant fluid is then decanted and is used as the stock luciferin solution. The oxidation of luciferin to oxyluciferin occurs spontaneously in the presence of oxygen in neutral and alkaline solutions, but in the absence of the enzyme such a spontaneous oxidation takes place without the production of light. This factor frequently plays a large part in the variations in the amount of light emitted from a given solution by reducing the available luciferin for the action of the luciferase. It has been customary, therefore, in the eleven series of experiments to use acid sea water in extracting the substrate, since acidity of the solution prevents the spontaneous oxidation. Later neutralization of the acid solution is effected in the actual experiments with N/10 NaOH, just previous to the addition of the enzyme.

In four series of experiments, another method of preventing spontaneous oxidation of luciferin was employed without the use of acid sea water. This method consisted of passing hydrogen through the luciferin solution for a considerable period of time immediately following the destruction of the enzyme. For this purpose a tall burette calibrated to 100 cc. was fitted with a rubber stopper at the open end. Through this stopper a U-shaped glass tube of small bore was fitted to allow the escape of gas. By connecting the stop-cock of the burette to a hydrogen tank the contained oxygen could be replaced by hydrogen previous to the preparation of luciferin. The latter was then quickly poured into the burette filled with hydrogen, as soon after boiling as possible. The stopper was reinserted in the tube and hydrogen allowed to bubble through the solution until the whole mass had cooled to approximately room temperature. This procedure, however, lessens the amount of light emitted from the solution as will be seen from the data to be presented later.

In still other experimental series the hydrogen method was employed with sea water which had been neutralized to a pH 7.0. Very satisfactory results were obtained with this method of preparing the substrate.

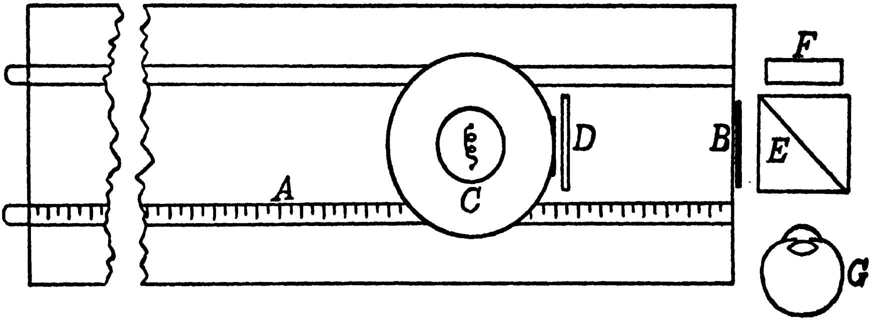

FIG. 2. Diagram of photometer bench. *A*, photometer bench; *B*, opal glass screen; *C*, chamber containing 21 C.P. lamp; *D*, No. 75 Wratten filter; *E*, comparison cube; *F*, mirrored vessel; *G*, position of observer.

The Photometric Method.

The measurement of luminescence of *Cypridina* solutions of varied concentrations is made possible by a comparison of the brightness of certain thicknesses of solution with the brightness emitted from a white surface illuminated by a light source of known intensity. The photometric method used in these experiments is an adaptation of that used by Harvey, 1925, and Morrison. The photometer bench (Fig. 2) consists of a track, one bar of which is marked off by divisions in mm. At one end of the bench (in this case at the 150 cm. mark) is placed a dull black screen with an opening in the center measuring 4.5 × 4.5 cm. square. Across this opening is fastened an opal glass screen. Care is taken that the screen shall be kept at the 150 cm. mark at all times. Behind this screen, on a movable standard, is a cylindrical dark chamber with an opening covered by a No. 75 Wratten blue green filter whose color value approximates most nearly that of the luminous solution of *Cypridina*. This chamber contains a small 6 volt 21 C.P. lamp of the type used in automobile headlights, so placed that illumination of the opal screen is complete. This chamber is attached to a sliding base so that the lamp can be moved toward or away from the screen. Finally a plain black screen is fastened at the lower end on the calibrated scale of the photometer bench and the whole apparatus is entirely covered with a photographer's black cloth to prevent any reflections or radiations from extraneous sources from registering on the opal glass. In front of the opal glass screen is placed a comparison cube.[1] This cube is so constructed that alternately mirrored bands on the hypothenuse of one prism deflect a source of light issuing at right angles to the line of vision of an observer, while at the same time a beam of light in line with his vision is transmitted directly through the cube. Thus two different sources of light would appear to an experimenter as parallel adjacent bands whose intensities can easily be compared with only slight observational errors due to personal equations.

The source of reflected light is the 6 volt 21 C.P. lamp on the photometer bench. The source of the transmitted light is the solution of *Cypridina* coming from a specially constructed rectangular vessel placed behind the comparison cube. This vessel is made of glass, the front of clear glass, the back of opal glass and the sides and bottom mirrored so that the lateral dimensions of the vessel may be approximately infinite. The vessel used was 2 cm. in thickness by inside measurement.

The photometer lamp was always run at 2.3 amperes and 4.8 volts. The transmission coefficient of the No. 75 Wratten filter measured[2] 0.00573 with the lamp at 4.8 volts. The photometer lamp was calibrated without the Wratten filter by measuring with a Macbeth illuminometer the illumination of the opal glass, when the lamp was set at definite distances and the inverse square law applied for other distances. The test plate used with the Macbeth illuminometer had been calibrated by the Electric Testing Laboratories and gave a reflection factor of 0.79. By the formula, foot candles × constant, 1.076 (foot candles to millilamberts) × 0.79 (reflection factor) × 0.00573 (Wratten filter transmission factor) one obtains the brightness of the opal screen in millilamberts.

EXPERIMENTAL.

The experiments were always conducted in a dark room and with the aid of an assistant. The procedure of a typical experiment is here recorded in some detail as a matter of ease in understanding the variation which will be referred to later. It was customary to use 100 cc. N/10 HCl acid sea water and 1 gm. of powder for the luciferin extract. 10 cc. of this solution were carefully measured in a calibrated pipette and poured into the rectangular mirrored vessel.

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[1] The comparison cube was kindly loaned by the Nela Research Laboratories of Cleveland.

[2] Kindly made by Dr. W. E. Forsythe of the Nela Research Laboratories for Dr. E. Newton Harvey.

To this was then added 5 cc. of sea water and 5 cc. of N/10 NaOH to neutralize the acid sea water. However, due to the buffers in the sea water and the alkaline nature of the *Cypridina* extract itself, the pH of such a solution ranged usually about 7.7. Such a solution contains about 0.1 gm. of *Cypridina* extract, although probably only a very small proportion of the powder used contains the luminous material, since the luminous organ comprises an extremly small percentage of the animal's body.

Immediately after preparing the vessel with the luciferin solution, 1 cc. of luciferase, containing 0.005 gm. of dry *Cypridina*, was quickly added to the luciferin solution and the time recorded. The vessel was rapidly agitated to facilitate mixing and placed immediately behind the comparison cube of the photometer apparatus. The observer then adjusted the photometer lamp until a match in intensities of the luminous bands on the comparison cube was obtained. At that moment the time in seconds and the distance in cm. on the bench scale were recorded by the assistant. The same procedure was repeated as the intensity diminished until the bands of light became too faint to read accurately. Very low intensities were discarded because of too great an error in comparison, and also frequently extremely high intensities were of little experimental value due to the fact that color differences between Wratten filter and the solution were accentuated.

TABLE I.

Series 3, Run 3.

Time	Distances (n)	n^2	$\frac{1}{n^2} \times 1000$	Logarithms
10	13.5	182.25	5.494	0.739
21	17.5	306.25	3.2679	0.514
31	22.0	484.00	2.0661	0.315
49	30.8	948.64	1.0548	0.023
65	45.8	2097.64	0.4784	$\bar{1}.679$
20	62.0	3844.00	0.2604	$\bar{1}.415$
36	87.8	7708.84	0.1298	$\bar{1}.113$
48	109.0	11881.00	0.0847	$\bar{2}.927$

Data calculated from observations in a typical experiment.

Since intensity varies inversely as the square of the distance, the data obtained from such a series of records are tabulated by squaring the distance figures, and then obtaining the reciprocals of these squares and plotting the reciprocals against time. The reciprocals are taken as the arbitrary units used in the calculations. The graph in Fig. 1 is plotted from the data in Table I. These curves are comparable to Amberson's in showing that light dies out rapidly and follows the form of a pseudomonomolecular reaction. If the logarithms of the intensities are plotted against time, a straight line is obtained.

Immediately after a series of runs in any given set of experiments, the hydrogen ion concentration was determined by the colorimetric method for each solution used.

In any luminous solution of definite thickness prepared in as crude a manner as the *Cypridina* extract considerable absorption and some scattering of light must occur, due to particles held in suspension. Harvey, 1925, found this factor a very important one to consider in determining the lumens emitted by his emulsion of luminous bacteria. After recording the pH values, all the solutions of the same concentration were poured together and the light transmission determined directly as follows. The rectangular glass vessel with the mirrored sides and bottom and the opal glass back, which had been used for the experiments themselves, was filled with water and placed in front of the photometer lamp with the Wratten filter in place. The Macbeth illuminometer, also fitted with a No. 75 Wratten filter on the lamp side, was placed in front of the vessel of water and the inverse square scale on the illuminometer set at 10. The apparatus was then adjusted until a match in the fields of the illuminometer was obtained. This match with pure water represents 100 per cent transmission. The water in the vessel was then replaced by the *Cypridina* solution to be measured, and without changing the position of the apparatus the illuminometer lamp was moved back and forth until a match of the fields was obtained. The inverse square scale reads directly, and consequently gives the percentage transmission of the *Cypridina* solution. Readings can be duplicated to about 5 per cent for the low concentrations used. Since the vessel used for measuring the transmission of the solution is 2 cm. in thickness, and since the purpose of the experiments is to determine the total light emitted from a 1 cm. cube of solution containing a definite amount of luminous powder, the thickness of the solution has to be taken into consideration and a correction made to obtain the transmission of a solution 1 cm. thick (T_1) by means of the formula

$$T_1 = \sqrt[n]{T_n}$$

in which T_n represents the thickness, n, of a solution used.

The determination of the amount of light emitted by 1 gm. of *Cypridina* powder is computed from the tabulated data as follows. It is necessary first to find the value in millilumens over 1 sq. cm. of the measuring vessel (2 cm. thick) for 1 arbitrary unit on the photometer scale, which will be constant for all experiments. If one obtains 2.1 foot candles of illumination of the opal glass for 2.5 arbitrary units, and since 1 millilambert in brightness equals 1 millilumen for every sq. cm., this value can be converted to millilumens per sq. cm. by the formula 2.1/2.5 foot candles × 0.00573, the transmission factor for the No. 75 Wratten filter, × 0.79, the reflection factor for the test plate, × 1.076, the conversion factor of foot candles to millilamberts. Solution of this formula gives 0.00408 millilumens per sq. cm. for 1 arbitrary unit. The total area of the decay curve (Fig. 1) is an index of the total amount of light emitted by the solution; this amount can be determined graphically by counting the squares and fractions of them. The straight line plotting of the logarithms is made to determine whether the experiment is progressing properly and also to determine the point of initial brightness of the solution at time 0. One square on the original plotting is equal to ½ an arbitrary unit for every 10 seconds, determined from actual readings (Fig. 1, lower left square), hence $k = 0.00408/2$ or 0.00204 millilumens per sq. cm. for 10 seconds. But this factor is the brightness for a solution measuring 2 cm. thick which shows some absorption. Correcting for 1 cm. and taking into account the transmission of the solution by the formula $T_1 = \sqrt[n]{T_n}$, where the transmission (T_2) in a typical experiment is 64 per cent, one obtains

$$T_1 = \sqrt{0.64}, \text{ or } \log T_1 = \frac{\log T_2}{2}$$

$$T_1 = 0.80 \text{ or } 80 \text{ per cent transmission.}$$

To determine the brightness of a solution 1 cm. thick with no absorption, $(\bar{B}_1)$, the formula

$$B_n = \frac{\bar{B}_1 (T_n - 1)}{\log e\ T_1}$$

is used, in which B_n is the brightness of a solution n cm. thick, and T_n is the transmission of a solution n cm. in thickness. Applying this formula to the typical experiment

$$0.00204 = \frac{\bar{B}_1 (.64 - 1)}{\log e\ .80} \text{ or } 0.00204 = \frac{\bar{B}_1 (-.36)}{-.223} \text{ or } \bar{B}_1 = 0.001263 \text{ millilumens per sq. cm.}$$

This value can be easily converted from a surface brightness in cm. squares to a cube emitting light in all directions by multiplying by 4, which gives 5.052×10^{-6} lumens, the value from 1 cc. of solution of perfect transparency calculated for 10 seconds and corrected for 64 per cent transmission. Taking 37.5 area squares or arbitrary squares derived from experimental plottings in a typical run, and solving for total light emission per gm. of *Cypridina* in a solution containing 0.01 gm. of powder per cc. of solution, one obtains

$$0.005052 \times 10 \times 37.5 = \frac{1.895}{0.01} \times 10^{-3}$$

or 0.1895 lumens per gm. of powder per second.

Effect of Luciferase Concentration upon Amount of Light.

Table II contains data from three series of experiments totalling 32 runs with different concentrations of luciferase. It is understood, of course, that in an impure solution such as luciferase the actual concentration of enzyme is undetermined, but the relative concentrations are known. The velocity constants as determined from curves plotted for these series are tabulated in Table II. In one case the ratios are almost exactly the theoretical value. Amberson's conclusion that "the velocity of reaction is very nearly proportional to enzyme concentration" is supported by the present work.

Inspection of the data from the records of lumen-seconds per cc. of *Cypridina* solution (squares) shows considerable variation. These variations in total light emission from solutions prepared in as nearly identical a manner as were the experimental solutions are not easily explained. Taking averages in all cases recorded, the total emission of light is about the same, but somewhat less, when the concentration

TABLE II.

Amount of Light and Enzyme Concentration.

Enzyme concentration (C) in cc.	k	$\frac{k_2}{k_1}$	Squares	T_2	T_1	$\bar{B}_1 \times 4$	Lumens	Averages
				per cent	*per cent*		*gm. per sec.*	
Experiment III								
1	1.5		33.5	64	80	5.075×10^{-6}	.3396	.356
1	1.875		37.5	"	"	"	.3806	
1	1.86	1.58	34.25	"	"	"	.3476	
2	3.0		35.5	"	"	"	.3603	.3121
2	2.5		27.0	"	"	"	.2740	
Experiment IV								
1	1.80		46.6	74	86	4.75×10^{-6}	.4427	.3844
1	1.55		40.6	"	"	"	.3857	
1	1.83		34.2	"	"	"	.3249	
2	3.87	2.11	37.4	"	"	"	.3553	.3591
2	2.70		38.6	"	"	"	.3667	
2	4.40		37.4	"	"	"	.3553	
Experiment VI								
1	1.17		39.3	72	84.8	4.107×10^{-6}	.3776	.3684
1	1.22		41.0	"	"	"	.3907	
1	1.075		35.37	"	"	"	.3369	
2	1.69	1.64	33.4	"	"	"	.3182	.3406
2	2.02		40.88	"	"	"	.3907	
2	2.02		33.0	"	"	"	.3229	

The constant (k) of the reaction velocity is determined from the slope of the logarithmic plotting.

k_2/k_1 represents the ratio of the average velocity reaction slopes where k_2 is the velocity constant, when double amounts of enzyme are used.

Each cc. of enzyme solution contained 0.005 gm. of dry *Cypridina* powder and was added to 20 cc. of luciferin solution.

T_2 and T_1 are transmissions for 2 cm. and 1 cm. thick solutions; $\bar{B}_1 \times 4$ is explained in the text.

of luciferase is doubled. This is seen in the total light emission recorded as lumens per second per gm. of dry *Cypridina* which for 5 runs averages 0.3777 for concentrations designated as *C* and 0.3507 for 2*C* concentrations of luciferase. If the amount of light were independent of the luciferase concentration, these values should be the same.

Effect of Luciferin Concentration upon Amount of Light.

Seven series of experiments totalling 60 runs were made with variations in the concentration of luciferin from 2 gm. to 0.5 gm. of powder per 100 cc. water. Acid sea water, neutral sea water and distilled water saturated with hydrogen were used to prepare the luciferin. The data of three typical series are tabulated in Table III. Again considerable variation occurs in the lumen-seconds (squares) emitted per cc. of solution with the same concentration of luciferase. With higher concentrations of luciferin, the squares of light emitted are about twice as great. The total light emission recorded as lumens per second per gm. of dry *Cypridina* averages about the same in the different experiments with some tendency for the weaker luciferin concentrations to give relatively more light.

With a constant concentration of enzyme the velocity constant should be the same for a wide range of substrate concentrations, and the straight line plottings should be parallel, or nearly so. This expected relationship, however, did not appear in any of the graphs. Similar anomalous results occur also in Amberson's records. It is obvious, then, that the luciferin-luciferase system represents an exceptional case of reaction velocity.

While this work primarily is concerned with the amount of light emitted from 1 gm. of *Cypridina* powder, it is, nevertheless, important to interpret the curves which indicate the nature of the enzyme reaction under investigation. Why do velocity constants vary for the different concentrations of substrate? Without doubt the oxidation of luciferin is a monomolecular reaction in a heterogeneous system, since luciferase is a colloid and probably a protein (Harvey, 1920). It not infrequently happens that the change in a chemical reaction is less, the greater the initial concentration of the substrate (Hoeber), hence the reaction velocity cannot be defined by a definite constant to signify the change in the two concentrations. Reactions of this sort are encountered in the inversion of cane sugar by invertase (Hudson), of grape sugar by zymase (Euler), and in the catalysis of H_2O_2 by platinum sol studied by Bredig and his students (Bredig and von Berneck). Hence the phenomenon is not peculiar to enzyme reactions

TABLE III.

Amount of Light and Substrate Concentration.

Substrate concentration in gm. *Cypridina* per cc.	k	$\frac{k_2}{k_1}$	Squares	T_2	T_1	$\bar{B}_1 \times 4$	Lumens	Averages
				per cent	*per cent*		*gm. per sec.*	
Experiment XI								
.005	2.4		41.5	59	76.8	5.273×10^{-6}	.4376	
“	2.54		40.3	“	“	“	.4249	.390
“	2.43		34.29	“	“	“	.3616	
“	2.72		32.0	“	“	“	.3374	
.0025	2.8	1.16	19.83	89	94.3	4.369×10^{-6}	.3465	
“	2.98		18.00	“	“	“	.3145	.3156
“	3.2		16.70	“	“	“	.2920	
“	2.8		17.71	“	“	“	.3095	
Experiment XVIII								
.0025	1.47		7.62	75	86.6	4.713×10^{-6}	.1436	
“	1.25		7.007	“	“	“	.1332	
“	1.35		7.33	“	“	“	.1382	.1398
“	1.36		7.66	“	“	“	.1444	
.00125	1.6	1.26	4.09	92	95.9	4.286×10^{-6}	.1402	
“	1.6		5.02	“	“	“	.1721	
“	1.73		4.42	“	“	“	.1515	.1519
“	2.0		4.20	“	“	“	.144	
Experiment XVII								
.01	1.17		92.08	74	86	4.75×10^{-6}	.4373	
“	1.04		83.39	“	“	“	.3961	
“	1.00		91.17	“	“	“	.4330	.417
“	0.9		84.54	“	“	“	.4015	
.005	1.5	1.53	44.20	84	91.6	4.464×10^{-6}	.3946	
“	1.58		54.59	“	“	“	.4874	.439
“	1.57		47.81	“	“	“	.4268	
“	1.48		50.12	“	“	“	.4474	

Symbols the same as in Table II.

alone, and seems to be associated with the physical nature of the reactants.

Study of the data of this paper gives no evidence that the reaction is altered greatly by variation in pH between 6.6 and 8.4. In one series, at pH 6.8 an average of 0.508 lumens was obtained, while a series run at pH 8.2 emitted an average of 0.445 lumens. Consideration of all the data obtained does not justify the conclusion that the differences in velocity are due solely to pH differences. Salts can be excluded from consideration, since in a solution made up with sea water the additions of small amounts of *Cypridina* solution would not greatly affect the total salt content. The solutions were always brought to a room temperature of about 20°C. Measurements of viscosity between solutions containing 10 cc. and 5 cc. luciferin respectively gave a difference in magnitude of approximately 1 per cent. This variation is negligible. All these factors can, therefore, be excluded as the cause of the anomaly in velocity constants for different luciferin concentrations.

In the work with catalysis of metal sols, the so called microheterogeneous systems, catalytic activity is increased in weaker substrate solution, whereas in macroheterogeneous systems where the dispersion is less extensive the reaction follows the theortical proportionality. It is quite probable that in suspensions and colloids the substrate adsorbs to the surface of the enzyme in greater degree from the more dilute solutions. So many factors are involved in the enzyme reactions among colloidal solutions that an exact formulation of the kinetics has never been satisfactorily made, but as a working hypothesis it may be assumed that the greater velocity constant with weak luciferin concentration is associated with greater adsorption from this concentration.

SUMMARY.

1. A photometric method was devised for measuring the intensities of light emitted per cc. of luciferin solution and calculating the amount of light emitted per gm. of dried *Cypridina* powder. A total of 128 runs was made and the data are incorporated in this report.
2. The maximum amount of light emitted from 1 gm. of powder under the experimental conditions was 0.655 lumens. Different samples of powder vary greatly in amount of light production.
3. When the concentration of substrate is doubled, nearly twice as

much light is emitted, or an average ratio $2C/C$ of 1.86. Calculations of total light emissions *per gm. of powder* at different concentrations indicate that slightly more light is produced from the smaller concentrations. The maximum amount of light was produced by the solutions made with neutral sea water and averaged 0.445 lumens. The least light was obtained from solutions in distilled water saturated with hydrogen. The technique allows too rapid spontaneous oxidation prior to the saturation with hydrogen. The maximum amount of light from such experiments was only 0.077 lumens Acid sea water solutions subsequently neutralized gave an average maximum of 0.386 lumens per gm. of powder per second.

4. When the concentration of enzyme is doubled, approximately the same amount of light is produced by both concentrations, although the stronger concentrations are slightly less effective than weaker ones. This undoubtedly is due to the colloidal nature of the enzyme and is a function of surface rather than of mass. In dilute solutions greater dispersion probably allows for greater adsorption to the surface of the enzyme. The average maximum amount of light produced in the series of enzyme experiments is of the magnitude 0.56 lumens per gm. of powder.

I wish to express my deep indebtedness to Dr. E. Newton Harvey under whose direction this study was undertaken, and to thank Mr. Stanton M. Hardy for the great help he rendered as my assistant.

I am also greatly indebted to Dr. Harvey for an abundant supply of *Cypridina hilgendorfii* Müller, the Japanese ostracod, the organism which has been used for the source of light in all the experiments.

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ON THE QUANTA OF LIGHT PRODUCED AND THE MOLECULES OF OXYGEN UTILIZED DURING CYPRIDINA LUMINESCENCE.

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The primary effect in a photochemical reaction is the absorption of a quantum $(h\nu)$[1] of light energy with the conversion of a single molecule into reaction products. Later, secondary effects, such as the production of a catalyst by the light, may obscure the quantum relation, resulting in much greater chemical change than 1 quantum per molecule On the contrary, not every quantum of light absorbed by a solution may result in chemical change, but there may be conversion into heat. In this case several quanta per molecule will be absorbed. As a matter of fact photochemical reactions are few in which the ratio of quanta absorbed to molecules decomposed is 1 (Taylor (1)).

In the reverse process, the emission of light through chemical change, chemiluminescence, we may also suppose as the primary process that 1 molecule undergoing change emits 1 quantum of light energy. Is light an invariable accompaniment of each molecular change? The few studies of chemiluminescence from this point of view show that change in many molecules occurs before 1 quantum is emitted. Thus, Haber and Zisch (2), studying the reaction of Na vapor and Cl at low pressures, observed the quanta of sodium *D* line emitted to be far less than the number of molecules of NaCl formed. This means that of the collisions between Na and Cl resulting in NaCl formation only a few emit light. It is obvious that this is an important point and should be studied further.

The present paper reports the results of an attempt to determine if a molecule of *Cypridina* luciferin, the oxidizable substance of the luminous crustacean, *Cypridina hilgendorfii*, emits 1 light quantum on oxidation in presence of luciferase. As luciferin cannot be obtained pure and consequently weighed out with accuracy, it is necessary to measure the oxygen rather than the luciferin used up during luminescence and to assume an oxidative dehydrogenation of luciferin, for which there is considerable evidence, thus:

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[1] A quantum of light is the constant, h, equivalent to 6.554×10^{-27} erg. sec., times the frequency, ν, of the light in question.

$$\text{luciferin} + 1/2\ O_2 = \text{oxyluciferin} + H_2O.$$

From this equation, which accounts for luminescence if luciferase is present, it is evident that 16 gm. or 3.03×10^{23} molecules of oxygen will oxidize a molecular weight of luciferin. If we measure the amount of oxygen consumed and the total amount of light produced by a given weight of *Cypridina*, the mg. of oxygen per lumen or the molecules of oxygen per quantum can be stated.

The total light produced is measured by direct photometric comparison of the brightness of a given depth of luminescent *Cypridina* solution with a surface whose brightness can be varied by known amounts. As the brightness of *Cypridina* luminescence decays with time, a curve of brightness against time is drawn, whose area will give the total amount of light produced in lumens, after allowing for absorption of light in the yellowish solutions. Details of the measurement are given by Stevens,[2] whose work shows that, with the same amount of luciferin, the total light is somewhat greater with smaller concentrations of luciferase. The total light is about proportional to the mass of luciferin but varies with other factors so that no great accuracy is claimed, even for samples of the same dried *Cypridina* material. In fact, only the order of magnitude of the oxygen per lumen can be given, since measurement of oxygen consumption presents the greatest difficulties.

The difficulty in measuring oxygen consumption by differential manometer methods is due to the fact that luciferin solution is prepared by making a boiling water extract (which destroys luciferase) of dried *Cypridina* and cooling the extract quickly. Such a solution is not in equilibrium with any *known* pressure of oxygen and cannot be brought into equilibrium with air without the oxidation of a considerable and unknown amount of luciferin without luminescence. It is therefore necessary to keep luciferin solution away from oxygen until ready to measure its oxygen consumption. The method finally adopted is to allow the luciferin luminescence to indicate the complete oxidation of the luciferin, as explained in connection with Fig. 1.

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[2] See preceding paper of this journal.

In Vessel *B*, of 25 cc. capacity, 4 cc. luciferase is placed (1 per cent dried *Cypridina* solution) and 0.5 cc. mercury to serve as a stirrer. With Cocks *F*, *C* and *D* open, and *E* closed, a stream of hydrogen freed of oxygen by passage over red hot platinized asbestos in a quartz tube is passed through the vessel for 1 hour, thus removing all oxygen. The hydrogen passes through lead tubing (since rubber tubing is permeable to oxygen) sealed to the *F* end of the apparatus with de Kohtinsky cement. Cocks *C* and *D* are then closed.

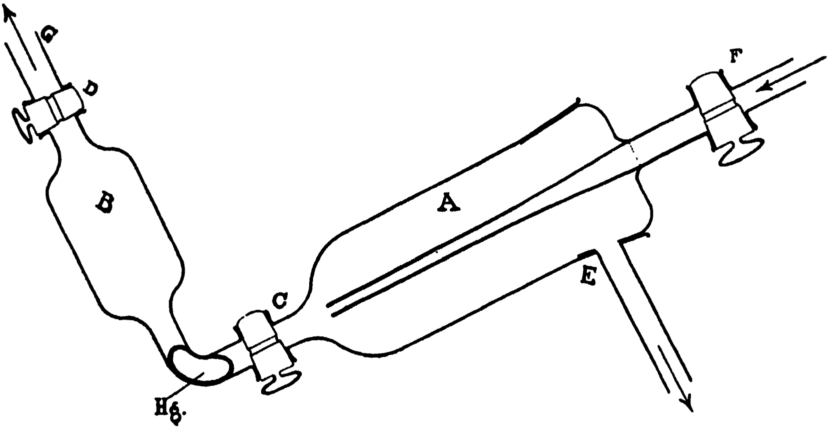

Fig. 1.

A solution of luciferin is then made by extracting 2 gm. of dry *Cypridina* with 50 cc. hot water (4 per cent solution) and immediately decanting into Vessel *A*. With Cocks *F* and *E* open, a stream of pure hydrogen is now passed through *A* for 1 hour, when *E* is closed. By opening *C* and *D* carefully, luciferin is now mixed with luciferase and *B* completely filled with fluid, when all cocks are closed and the vessel disconnected from the supply of hydrogen. There will be no luminescence in Vessel *B* since oxygen is absent.

The remaining luciferin in Vessel *A* can now be taken to the photometer bench, mixed with luciferase in presence of oxygen and the total light produced measured as described.[8] In a typical satisfactory experiment it amounted to 0.101 lumens per gm. of dried *Cypridina* material.

Vessel *A* is then filled with distilled water saturated with air at 22°C. Each cc. of distilled water will dissolve 0.006 cc. oxygen (measured at N. T. P.) By opening Cocks *C* and *D*, small amounts (0.1 to 0.2 cc.) of water are allowed to flow into *B*, displacing a corresponding amount of luminescent solution into *G* which can then be measured in *G*. After each admission the fluid in *B* is thoroughly mixed by shaking the mercury back and forth and luminescence noted. Luminescence following each admission of oxygen becomes less and less bright and is quite faint after 1 cc. water has been added, making allowance for the 0.1 cc. capacity of the bore of Stop-cock *C*, which contained no oxygen.

In a typical experiment 1.1 cc. water was admitted before luminescence ceased. To calculate the gm. of oxygen admitted: 1.1 cc. $\times$ 0.006 = 0.0066 cc. $\times$ 0.00143 = 9.44×10^{-6} gm. of oxygen were necessary to oxidize the luciferin in 20.5 cc. solution containing 4 per cent or 0.82 gm. dry *Cypridina*. Hence $9.44/0.82 = 11.5 \times 10^{-6}$ gm. of oxygen were used per gm. of dry *Cypridina* material. The total lumens per gm. of dry *Cypridina* material was found to be 0.101 in this experiment, as stated above. Hence $11.5 \times 10^{-6}/0.101 = 11.4 \times 10^{-5}$ gm. oxygen per lumen.

It is quite obvious that this method of determining oxygen cannot give very accurate results since the end-point is not very sharp. It is also certain that when the admission of oxygen results in only faint luminescence there must be an excess of oxygen present. This is because luminescence intensity is really dependent on reaction velocity and not on concentration of luciferin (Amberson (3)). With considerable luciferin and little oxygen, luminescence will be faint because the low oxygen concentration is slowing reaction velocity; with little luciferin and considerable oxygen, luminescence will be faint because the low luciferin concentration is slowing the reaction velocity. The latter statement corresponds to the condition when we are nearing the end-point in admitting oxygen. In fact it is possible to show that after 1 cc. of water has been admitted to Vessel *B*, there is excess oxygen present, since fresh oxygen-free luciferin solution introduced into *B* will give a fair luminescence.

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[8] I am deeply indebted to Mr. K. P. Stevens for making these measurements.

The results tell us only that less than 11.4×10^{-5} gm. oxygen per lumen are necessary for a lumen of luminescence. It will be noted also that the assumption is made that all the oxygen admitted to the luciferin solution is used in oxidizing luciferin, and that there are no other easily oxidizable compounds present. I have endeavored to test this point by adding to the dried *Cypridina* powder an equal weight of dried powdered pill-bugs (*Oniscus*) which might supply easily oxidizable substances but not luciferin, and then determining oxygen consumption. The runs with added dried *Oniscus* showed about 25 per cent more oxygen consumed, so that this factor can play no very great part in the oxygen consumption. However, the most that can be claimed is a determination of the order of magnitude of oxygen consumption per lumen of light emitted.

The average of eleven experiments completed without mishap was 10.6×10^{-5} gm. of oxygen per lumen, with extreme variations of 5×10^{-5} and 15.5×10^{-5} gm. per lumen. The average of five of the later more satisfactory experiments was 11.7×10^{-5} gm. per lumen with a maximum variation from the mean of 25 per cent.

Upon the basis of 11.4×10^{-5} gm. of oxygen per lumen of luminescence, we may calculate the molecules of oxygen per quantum with the aid of the visibility of radiation curve.[4] The energy distribution in the *Cypridina* luminescence spectrum has recently been determined by Coblentz and Hughes (4). The maximum emission is at $\lambda = 0.48\mu$ and the visibility curve tells us that 1 lumen of $\lambda = 0.48\mu$ light is equivalent to 0.01 watt or 0.01×10^7 ergs per second. A quantum ($h\nu$) of $\lambda = 0.48\mu$ light is equal to 4.1×10^{-12} ergs so that 1 lumen of $\lambda = 0.48\mu$ light contains 2.45×10^{16} quanta. As 11.4×10^{-5} gm. of oxygen contain 2.16×10^{18} molecules and will oxidize 4.32×10^{18} molecules of luciferin we see that 88 molecules of oxygen or 176 molecules of luciferin must undergo change in order to produce 1 quantum of light of $\lambda = 0.48\mu$.

For reasons stated above the actual value is probably somewhat less than this. A conservative statement might be that about 50 molecules of oxygen or 100 molecules of luciferin react to produce 1 quantum of *Cypridina* luminescence. *Certainly more than 1 molecule of oxygen per quantum is necessary.*

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[4] See data on light units, Trans. Illuminating Engineering Soc., 1922.

Accepting 50 molecules of oxygen per quantum or 6.48×10^{-5} gm. per lumen of luminescence, a few other orders of magnitude can be calculated, based on the luminescence equations (Harvey (5)):

$$\text{luciferin } (LH_2) = \text{oxyluciferin } (L) + H_2 - 15 \text{ Cal.}$$

$$H_2 + 1/2\ O_2 = H_2O \text{ (liquid)} + 69 \text{ Cal.}$$

The heat of oxidation of luciferin is therefore 54 Calories per gm. mol, justified on the general rule for heats of combustion of organic compounds (Thornton (6)). The gm. molecular heat equivalent of the quantum of $\lambda = 0.48\mu$ light is 59.3 Calories, obtained from the relation U (in Calories) = N (Avogadro constant) $h\nu$, but we are as yet uncertain whether this relationship can be applied to chemiluminescent reactions.

If 16 gm. of oxygen oxidize luciferin with production of 54,000 calories of heat, 6.48×10^{-5} gm. of oxygen will evolve 2.19×10^{-1} calories and correspond to the emission of 1 lumen of $\lambda = 0.48\mu$ light, equivalent to 2.39×10^{-3} calories. Hence the efficiency, *energy in light/heat of reaction*, is about 1.1 per cent. The rise in temperature in a 4 per cent solution of dried *Cypridina* should be in the neighborhood of 0.001°C.

The oxygen necessary to combine with the luciferin in 1 cc. of a 4 per cent solution of dried *Cypridina*, on the basis of 50 molecules per quantum, is approximately 0.3×10^{-6} gm. or a 0.00001 molecular solution. A 4 per cent *Cypridina* solution must therefore contain luciferin in approximately 0.00002 M concentration.

The results show beyond any doubt that more than 1 molecule of luciferin must react to produce 1 quantum. Luminescence is not therefore an invariable accompaniment of luciferin oxidation. This is to be expected if luciferase is the source of the light, which results from transfer of the energy of oxidation of luciferin to some of the luciferase molecules, exciting them to luminescence (Kautsky and Zocher (7)). Spontaneous oxidation of luciferin is proceeding and only those molecules of oxidizing luciferin can transfer their energy which occupy special positions as regards the luciferase molecules.

SUMMARY.

A study of the oxygen consumed per lumen of luminescence during oxidation of *Cypridina* luciferin in presence of luciferase, gives 11.4 × 10^{-5} gm. oxygen per lumen or 88 molecules per quantum of $\lambda = 0.48\mu$, the maximum in the *Cypridina* luminescence spectrum. For reasons given in the text, the actual value is probably somewhat less than this, perhaps of the order of 6.48 × 10^{-5} gm. per lumen or 50 molecules of oxygen and 100 molecules of luciferin per quantum. It is quite certain that more than 1 molecule per quantum must react.

On the basis of a reaction of the type:

$$\text{luciferin} + 1/2\ O_2 = \text{oxyluciferin} + H_2O + 54\ \text{Cal.},$$

it is calculated that the total efficiency of the luminescent process, *energy in luminescence/heat of reaction*, is about 1 per cent; and that a luciferin solution containing 4 per cent of dried *Cypridina* material should rise in temperature about 0.001°C. during luminescence, and contain luciferin in approximately 0.00002 molecular concentration.

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THE KINETICS OF OSMOSIS.

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The equilibrium conditions in osmotic pressure experiments have been thoroughly investigated both theoretically and experimentally. The kinetics of the process, however, has received little attention although the general theory is known. The process of osmosis is essentially one of diffusion of the solvent into the solution and so should follow in general the diffusion laws. In ordinary diffusion experiments, however, such as the solution of a solid or the diffusion of salt through a vessel of water, the solute is the component which moves while in osmosis it is the solvent. In the former case, since the number of solute molecules is ordinarily only a small fraction of the total number, the total number of molecules remains practically constant, while in osmosis, the total number of molecules in the solution changes during the experiment. It might be expected therefore that the equation for osmosis would differ slightly from that of diffusion of the solute since the terms containing the total number of molecules, *i.e.* the volume, which are constant in the ordinary diffusion formula are now variables. As will be seen this is the case experimentally.

The desired relation may be derived in a number of ways, but the following derivation, although not mathematically rigorous, appears to the writer to be the simplest.

Assume the solution separated from the solvent by a membrane permeable only for the solvent, as shown in Fig. 1. The mole fraction of the solute is assumed small and the solution is assumed to obey the laws of ideal solutions Solvent will pass through the membrane from the pure solvent into the solution. The volume and hence the hydrostatic pressure on the solution will be increased and the process will stop when the hydrostatic pressure equals the osmotic pressure. It is desired to know the quantity of solvent which passes through the membrane at any time.

According to the general law of diffusion (or flow) the rate of flow per unit area is proportional to the pressure gradient, or, in this case, to the pressure divided by the resistance; or

$$\frac{dv}{dt} \sim \frac{P}{R_1},$$

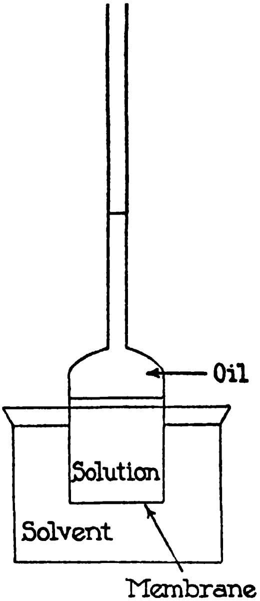

FIG. 1. Apparatus for the determination of the rate of osmosis.

in which v is the volume, t the time, P the pressure, and R the resistance offered to the flow.

In order to integrate this equation the variable terms must be expressed as functions of v or t. The pressure is evidently equal to the osmotic pressure, which tends to force the water in, less the hydrostatic pressure, which tends to force it out,

$$P = OP - HP.$$

The osmotic pressure is defined by the equation,[1]

$$OP = \frac{RT}{V_m}(-\ ln(I - x))$$

which for dilute solutions reduces to

$$OP = \frac{RTx}{V_m} \tag{1}$$

where R is the gas constant, T the absolute temperature, x the mole fraction of the solute, and V_m the molal volume of the solvent in the solution.[2] Assume there are g gm. of solute of molecular weight M dissolved in V cc. of water. The mole fraction of the solute, then, will be the moles of solute divided by the total moles, or

$$x = \frac{\frac{g}{M}}{\frac{V}{V_m} + \frac{g}{M}}.$$

Since $\frac{g}{M}$ is assumed small in comparison to $\frac{V}{V_m}$, the equation may be written $x = \frac{gV_m}{MV}$. Substituting this value of x in (1),

$$OP = \frac{RT}{V_m} \cdot \frac{gV_m}{MV},$$

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[1] *Cf.*, for instance, Washburn, E. W., Physical chemistry, New York, 1st edition, 1915, 155.

[2] Since we are interested only in the *amount* of water that diffuses into the system, *i.e.*, the amount that passes through the plane at the outside surface of the membrane, it is not necessary to consider the pressure gradient within the solution. The pressure may therefore be assumed proportional to the average pressure, *i.e.*, to the pressure that would exist if the solution were homogeneous. That this is so may be seen from the fact that the pressure gradient would depend on the diffusion coefficient, which does not affect the form of the equation but only the value of the constant. Mathematical proof of this statement may be found in the fact that if the equation is solved according to the general form of Fouriers theorem, which takes into account the pressure gradient and gives the amount of water which passes through a plane at any distance y, from the surface of the membrane, and y is then made equal to zero, the equation reduces to the same form as the integral of (1). *Cf.* Mellor, J. W., Higher mathematics for students of chemistry and physics, New York, 4th edition, 1913, 488.

or, since in any one experiment R, T, M and also g are all constant and may be combined into one constant P_o,

$$OP = \frac{P_o}{V}. \tag{2}$$

The hydrostatic pressure will be equal to the initial pressure, n', plus the additional pressure caused by the rise of the solution in the capillary. If 1 cc. increase in volume causes the liquid to rise K' mm., and f is the relative specific gravity of the liquid compared to mercury, then the hydrostatic pressure at any time expressed in mm. of mercury will be equal to $fn' + fK' (v - v_o)$, or $HP = n + K (v - v_o)$, where v is the volume at any time and v_o is the initial volume; n is the initial pressure expressed as mm. mercury; and K is the increase in pressure per cc. increase in volume, expressed also as mm. mercury.

Evaluation of R_1.— R_1 may be expressed in different ways depending on the mechanism assumed for the passage of water through the membrane. If the water is assumed to dissolve in the membrane and so pass by diffusion, R_1 is a function of the diffusion coefficient. If the water is assumed to flow through capillaries, then R_1 is a function of the size and number of the capillaries.

1. The Water Dissolves in the Membrane.

The thickness of the layer of solution for a cylindrical vessel with the membrane at one end will be $\frac{v}{\pi r^2}$, where r is the radius of the cylinder. If the thickness of the membrane is h then the total average distance the water has to diffuse will be $h + \frac{v}{2\pi r^2}$, and the total resistance offered to its flow per unit of area will be the distance times the specific resistance; or if R_m is the resistance offered by unit thickness of collodion and R_w the resistance offered by unit thickness of the solution, the total resistance $R_1 = hR_m + \frac{v}{2\pi r^2} R_w$. If the membrane is of such a nature therefore that the solvent can diffuse through it as rapidly or nearly so as through the solution, it is evident that the resistance offered by the membrane may be neglected, since the distance passed through in the membrane is very small compared to the total distance, and $R = \frac{v}{2\pi r^2} R_w$. In the case of collodion membranes and most other artificial membranes the resistance offered by the membrane is enormously greater than that offered by the solution, so that the term representing the resistance of the solution may be neglected and $R = hR_m$. Since the diffusion coefficient is the reciprocal of the specific resistance, $R = \frac{h}{C}$ where C is the diffusion coefficient of the solvent in the membrane.

2. *The Water Flows through Capillaries in the Membrane.*

In the evaluation of the resistance given above the solvent was assumed to diffuse through the membrane in the same way as through the solution and the increase in resistance was ascribed to the difference in the rate of diffusion of the solvent molecules in the membrane and in the solution. There is some reason to believe, however, that collodion membranes at any rate may be considered as consisting of pores in a solid and that the water passes only through the pores. From this point of view the resistance offered by the membrane will be determined by Poiseuille's law.[3] The resistance offered by the solution can again be neglected. If there are p pores of radius r_1 per unit of surface, and they are assumed to be the same length as the thickness of the membrane, the quantity of water that will pass under unit pressure according to Poiseuille's law will be proportional to $\frac{pr_1^4}{h\eta}$, η being the viscosity, and the resistance offered to the passage of the water will be the reciprocal of this or $\frac{h\eta}{pr_1^4}$. Since for any one solvent and membrane η, p and r_1 are constant the resistance will be $\frac{h}{C}$ as before.

In either case, then, the total amount of water passing through will be proportional to $\frac{SC}{h}$ where S is the total surface of the membrane.

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[3] *Cf.* Hitchcock, D. I., *J. Gen. Physiol.*, 1925–27, viii, 71.

Substituting these values of R and P, equation (1) becomes

$$\frac{dv}{dt} = \frac{CS}{h}\left[\frac{P_o}{v} - [n + K(v - v_o)]\right]. \tag{3}$$

C is the quantity of solvent that will pass through a unit area of membrane of unit thickness in unit time under unit pressure. Changes in the value of C are due then either to changes in the rate of diffusion in the membrane or to changes in the pore size or number, or the viscosity of the solvent, depending on which mechanism is assumed for the passage of the solvent through the membrane.

If there is no hydrostatic pressure on the solution at the beginning of the experiment the equation in this form predicts that the quantity of solvent passing through the membrane in the first few minutes will be proportional to the osmotic pressure of the solution. This relation has been shown to be true by a number of workers and has been used to measure the pressure in cases where the equilibrium value could not be obtained.[4]

At equilibrium no solvent passes through the membrane, *i.e.* $\frac{dv}{dt} = 0$, so that

$$\frac{P_o}{v_e} = n + K(v_e - v_o), \tag{4}$$

or

$$Kv_o = n + Kv_e - \frac{P_o}{v_e},$$

where v_e is the volume of solution at equilibrium.

Equation (3) may be integrated in a number of forms depending on which constants are used. Mathematically the simplest expression is obtained in terms of P_o, v_e and K. In order to obtain the equation in these terms the value of Kv_o, from equation (4), is substituted in equation (3). Collecting terms and simplifying, the equation becomes $\frac{dv}{dt} = \frac{CSP_o}{hv_e}\frac{(1 + bv)(v_e - v)}{v}$, where $b = \frac{Kv_e}{P_o}$, which, on integration, gives

$$C = \frac{2.3\, hv_e}{(1 + bv_e)SP_o t}\left(v_e \log\frac{v_e - v_o}{v_e - v} - \frac{1}{b}\log\frac{1 + bv}{1 + bv_o}\right); \tag{5}$$

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[4] *Cf.* Findlay, A., Osmotic pressure, London and New York, 2nd edition, 1919.

or if $K = 0$, that is when the experiment is so arranged that the hydrostatic pressure is constant,

$$C = \frac{hv_e}{SP_o t}\left(v_o - v + 2.3\, v_e \log \frac{v_e - v_o}{v_e - v}\right). \tag{6}$$

TABLE I.

Rate of Osmosis 30°C.

Experiment 1.

$v_o = 3.0$ $P_0 = 52.5$ $K = 2.28$ $n = 1.1$ $v_e = 6.2$ $b = .27$
$S = 10$ sq. cm.

T	v	$K_m \times 10^4$	$\frac{C}{h} \times 10^4$
hrs.	*cc.*		
0	3.0		
24	3.70	47.5	2.27
48	4.20	44.0	2.19
96	4.85	38.0	1.96
192	5.60	36.0	1.98

Experiment 2

3.2 cc. a "soluble" gelatin in thistle tube closed with collodion membrane.
$v_o = 3.2$ $P_o = 288$ $K = 0$ $n = 50$ $v_e = 5.85$ $S = 4.5$ sq. cm.

T	v	$K_m \times 10^4$	$\frac{C}{h} \times 10^4$
hrs.	*cc.*		
0	3.2		
24	3.75	42.5	1.53
48	4.15	40.0	1.53
96	4.72	38.7	1.62
192	5.22	32.0	1.51
300	5.85		

This condition would also be true if the solvent outside the membrane were replaced by a large volume of solution having a lower osmotic pressure than that of the solution inside. The osmotic pressure of the outside solution would enter into the equation in the same way as does the initial hydrostatic pressure, n, in equation (3).

A number of experiments were performed to test the accuracy of these equations, and they were found to hold within the experimental error.

Table I and Fig. 2 give the results of two such experiments. In the first experiment solutions of egg albumin in M$(NH_4)_2SO_4$ were placed in a rocking osmometer,[5] with the same concentration of ammonium sulfate outside, and left until equilibrium was established.

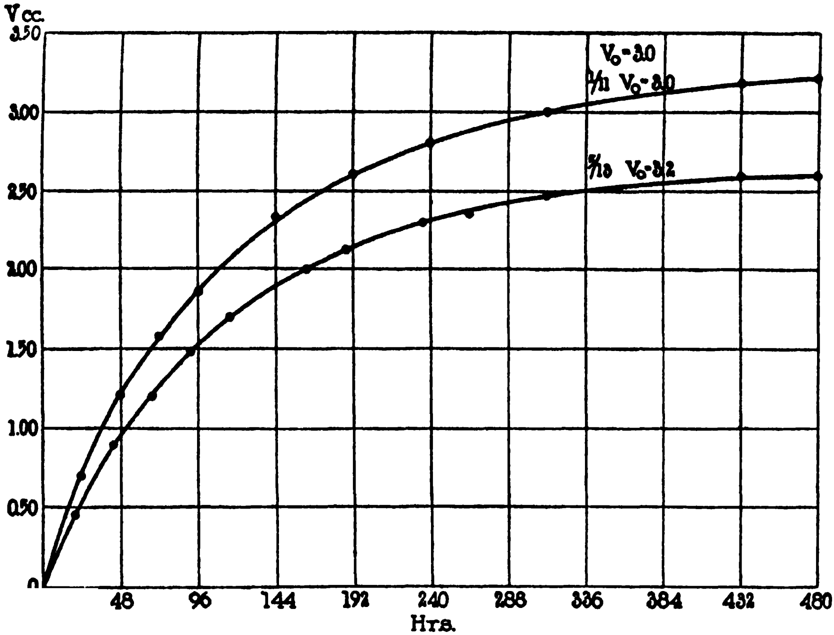

FIG. 2. The rate of osmosis of protein solutions in collodion membranes.

3 cc. of the solution was then placed in a tube closed with a membrane, the upper part of the tube filled with oil and the membrane immersed in the solution of ammonium sulfate with which the albumin solution had previously been in equilibrium. The pressure in the manometer was set at the equilibrium value and the system left for 2 days so that the permeability of the membrane might become constant. The manometer level was then lowered and the rise of the

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[5] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 351; also 1926–27, x, 161.

oil in the manometer noted. The experiment was carried on in a water bath at 30°C. In the presence of this concentration of ammonium sulfate the osmotic pressure of albumin is nearly proportional to its concentration, so that it may be assumed to obey the ideal solution law. The ammonium sulfate also prevents bacterial growth. The second experiment was done in the same way except that a solution of "soluble" gelatin[5] was used. In this case the manometer tube was bent so as to be horizontal. There was therefore no change in pressure during the experiment and equation (6) should fit. The table shows in both cases that the monomolecular constant K_m given for comparison shows a regular decrease while the constants calculated by equations (5) and (6) do not vary outside of the experimental error. This was found to be the case in all of the experiments made. The monomolecular constant dropped slowly in every case.

In the first experiment the value of $\frac{C}{h}$ was found to be 2×10^{-4}. If the derivation given is correct this should be the cc. of water that will flow through 1 sq. cm. of the membrane in 1 hour under 1 mm. mercury pressure. At the end of the experiment the membrane was washed, filled with water and the rate of flow of water through it determined under 10 cm. mercury pressure. A value for the rate of flow of 1.5×10^{-4} cc. per hour per mm. mercury pressure was obtained, which agrees as well as could be expected with the figure calculated from the osmotic pressure experiment.

In this experiment the surface of the membrane is constant. In experiments with cells such as those of McCutcheon and Lucke[6] the surface increases during the experiment. If the water is assumed to diffuse through the membrane, the thickness of the membrane being constant, then $S \propto v^{\frac{2}{3}}$; or if the volume of the membrane remains constant $\frac{S}{h} \propto v^{\frac{4}{3}}$. If the water is supposed to flow through pores in the membrane and the increase in surface is due to enlarging the size of the pores, the thickness remaining constant, then $r^2 \propto S \propto v^{\frac{2}{3}}$ and $r^4 \propto v^{\frac{4}{3}}$. According to the first assumption the velocity should be very slightly slower than that predicted by the monomolecular formula and according to the second or third assumption it should be very slightly faster. In either case the deviation from the monomolecular formula would be noticed only in very accurate experiments.

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[6] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1925–26, ix, 697.

SUMMARY.

It is shown that by combining the osmotic pressure and rate of diffusion laws an equation can be derived for the kinetics of osmosis.

The equation has been found to agree with experiments on the rate of osmosis for egg albumin and gelatin solutions with collodion membranes.

THE SWELLING OF ISOELECTRIC GELATIN IN WATER.

I. Equilibrium Conditions.

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If a block of isoelectric gelatin is placed in water it will imbibe water and increase in volume. The writer and Kunitz (1) were able to measure the pressure with which this water was drawn into the block and found that it increases with the concentration of the gelatin. According to the phase rule the concentration (osmotic pressure) of a solution in the presence of the solid is independent of the amount of solid. It was suggested therefore that gelatin consisted of two or more fractions some of which were insoluble at low temperature while others were soluble and so could exert osmotic pressure. The swelling of isoelectric gelatin then becomes a process of osmosis just as Wilson (2) showed was the case for the swelling of gelatin in acid, except that in the case of isoelectric gelatin the osmotic pressure is not due to the ions of an electrolyte but to the presence of a soluble constituent of the gelatin itself. Water therefore enters the gelatin until the elastic pressure is equal to the osmotic pressure. The present paper is an attempt to apply this mechanism quantitatively to the swelling of gelatin.

The general behavior of gelatin when placed in water has been described by a number of investigators and in detail by the very complete experiments of Arisz (3). The more striking peculiarities may be briefly described as follows. In general the swelling increases with the temperature and with the concentration of gelatin. A block of gelatin concentrated by allowing the water to evaporate swells much more than a similar block made by allowing a solution of the same concentration to solidify. Thin films of gelatin reach a value which increases only slowly while large blocks do not give any indication of a maximum value but continue to swell until dissolved. At higher temperatures there is less indication of an equilibrium value. If a block of gelatin is allowed to remain in water until it has stopped swelling and then is raised to a higher temperature in air for a short time under such conditions that there is no change in volume, it will swell rapidly when replaced in water at the first temperature. These peculiarities are similar to those of any substance when under an elastic strain, and can be readily accounted for at least qualitatively by the mechanism of swelling stated above. When the block is placed in water, water enters owing to the osmotic pressure of the solution in the block. The fibres of solid material are thereby forced apart and the force with which they attempt to return to their original position opposes the entrance of the water. At the same time the osmotic pressure is decreasing owing to dilution. When the elastic force equals this osmotic pressure the process stops. If the fibres have been stretched beyond their elastic limit, however, or if the force is applied for too long a period of time the fibres become fatigued and the elastic force is lessened so that more water enters. As is the case with any elastic body therefore a true equilibrium value is never reached. The osmotic pressure increases with the temperature and at the same time the amount of solid material decreases and also probably becomes less elastic so that swelling increases very rapidly as the temperature rises. In a thin film the whole mass becomes filled with water before the fibres become fatigued whereas in a large block the outside layers, which swell first, become fatigued and take up more water before the water has diffused into the inner layers. This "secondary swelling" therefore overlaps the primary so that in the case of large blocks there is no indication of a maximum value. This mechanism will be discussed more in detail under the kinetics of the swelling process. When a block which has swollen at a low temperature is raised to a higher temperature the elasticity is destroyed and it therefore swells again when replaced in water. When the gelatin is concentrated by evaporation it decreases in size and is therefore under an elastic strain just as when it swells except that in the case of loss of water the elastic force is in the same direction as the osmotic pressure instead of opposite to it. The block therefore swells more than a similar one which is not under elastic strain at this concentration.

Experimental Procedure.

In order to avoid the difficulty of working with very thin pieces of gelatin the gelatin was coated on glass rods about 0.2 by 15 cm. The rods were weighed, dipped in gelatin of the desired strength at a temperature of 40°C., weighed again in order to determine the amount of gelatin in the film and then placed in stoppered tubes containing a strip of wet filter paper in order to prevent loss of water by evaporation.[1] The tubes were then put in the cold room at 5°C. Arisz found that gelatin swells more if placed in water at a low temperature immediately after solidifying than if kept at the same temperature in air for a time before placing in water. After the 1st day no further change occurred. These experiments were repeated and confirmed except that a change was noted for the first 3 days. All the gelatin used in these experiments was therefore kept at 5° for 3 days before it was placed in water. If, as the writer assumes, solid gelatin contains a saturated solution of the material forming the network this is exactly the behavior expected since time is required to reach the equilibrium value. The experiment is analogous to that of Loeb (5) who found that the osmotic pressure of a gelatin solution cooled from 70° to 37° was higher at first than that of a solution raised from 10° to 37°, but later became the same. At the beginning of an experiment the rods were weighed again and then placed in water at 5° and weighed at intervals after wiping dry with soft filter paper. Since warming even for a short time destroys the elasticity, it is necessary to weigh at the same temperature as that at which the swelling occurred. Some experiments were also made with slides dipped in gelatin and with thin slices of gelatin cut from a cylinder. Spheres of gelatin made by dropping liquid gelatin into cold toluene were also used.

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[1] Schroeder found that gelatin which was in apparent equilibrium with water lost weight when suspended in saturated water vapor at the same temperature and this has been considered as contrary to the second law of thermodynamics. As Bogue (4) has pointed out, however, the force of gravity is acting on the gelatin in the air but not in the water and if the gelatin is supposed to consist of a network of capillaries, this is sufficient to account for the observation. Suppose a fine capillary is dipped into water and after the water has risen in the tube, the tube is removed and suspended vertically just above the surface of the liquid. A drop will be formed at the bottom of the tube and since this drop has a convex surface its vapor pressure will be higher than that of the body of water. Water will evaporate from the drop until the surface of the water at the bottom of the capillary is no longer convex.

For convenience in the calculation and presentation of the results they have been expressed in terms of gm. of water per gm. of gelatin.

Measurement of the Swelling Pressure.—The pressure with which the water tends to diffuse into the gelatin was measured by the writer and Kunitz (1). The measurements were extended to higher concentrations using the same method as described. The results of these

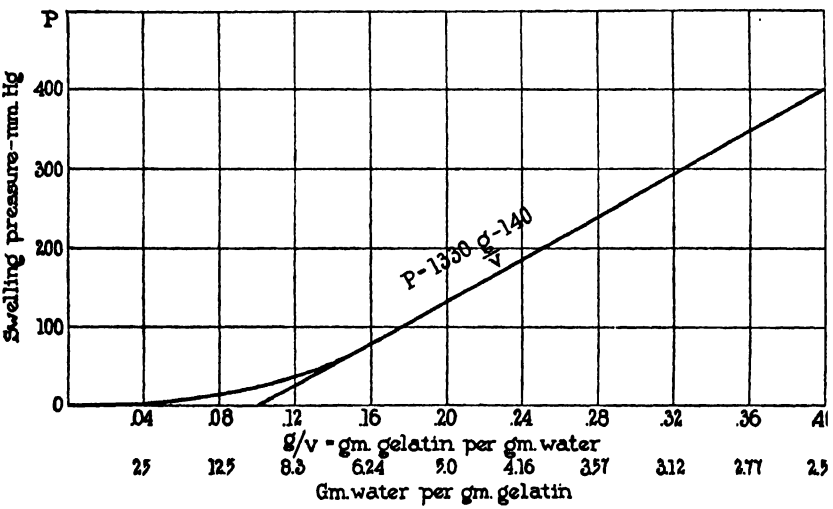

FIG. 1. Concentration-swelling pressure of gelatin at 10°.

measurements of the swelling pressure of isoelectric gelatin at 5° are shown in Fig. 1.

These pressures were obtained with gelatin that had been made up to the concentration noted while liquid and it seemed quite possible that a different pressure would be obtained when the concentration was changed by a swelling of the solid gelatin. In order to test this point a Chamberland filter was coated with collodion and then with gelatin containing 40 gm. per 100 gm. of water. The thimble was then placed in water for 12 hours at the end of which time the concentration was 30 gm. of gelatin per 100 gm. of water. The filter and gelatin were then removed from the water, the inside filled with water and a manometer tube attached as previously described (1). At first no pressure was obtained as the swelling pressure was balanced by the elasticity of the gelatin. As the elasticity decreased with time, however, the pressure gradually rose and after 4 days remained constant at 255 mm.Hg pressure. This is the same, within the experimental error, as the value obtained with 30 per cent gelatin which had been prepared by adding water to the melted gelatin. The effect on the osmotic pressure caused by the addition of water is therefore the same whether the water is added to the solid or liquid gelatin.

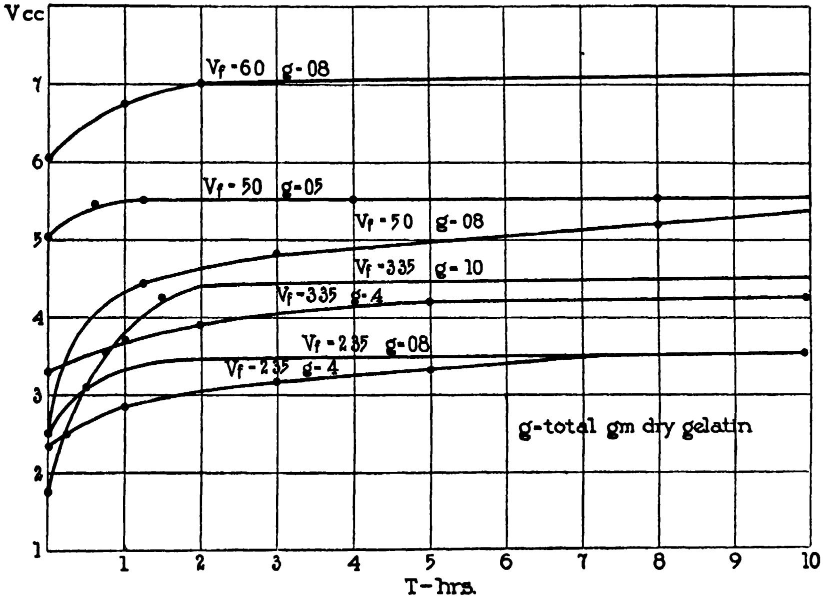

FIG. 2. Swelling of various concentrations of gelatin in water at 5°.

The results of some typical swelling experiments are shown in Fig. 2. It is evident that at this temperature and with thin films of gelatin, a fairly constant maximum value is reached. The experiments also show that the final value for the swelling depends only on V_f, the concentration of the gelatin at the time it solidified and, within the limits of variation used in these experiments, is independent of the concentration of the gelatin when it is put in water.

Calculation of the Equilibrium Conditions.

According to the mechanism of swelling outlined above equilibrium is reached when the osmotic pressure equals the elastic pressure. The elastic pressure in this case takes the place of the hydrostatic pressure in the case of osmosis experiments discussed in a preceding paper (6). At equilibrium then

$$OP = EP \tag{1}$$

In order to use this relation both pressures must be expressed in terms of the volume of water In ideal dilute solutions the osmotic pressure may be written $OP = P_o/V$ where P_o is a constant depending on the concentration and molecular weight of the solute, the molal volume of the solvent and the temperature, and V is the gm solvent. It has been shown by Kunitz (7) that the osmotic pressure of gelatin solutions also obeys this law when the proper correction is made for the amount of solvent combined with the gelatin. The relation is rather complicated, however, and it is more convenient to use an empirical relation. It may be seen from Fig. 1, that at concentrations of between .1 and .4 gm. gelatin per gm. of water, the swelling pressure curve may be represented by the equation $P = \frac{1330g - 140v}{v}$ where g is the gm. gelatin and v the gm. water or $P = \frac{1330 - 140\,V}{V}$ where V is the gm. water per gm. gelatin.

The bulk modulus of an elastic body by definition is proportional to the elastic force divided by the difference between the volume when under no strain and the volume under the force applied. Or in terms of the original volume, it is the force required to increase the volume by an amount equal to the original volume.

$$K_e = \frac{EP\ V_f}{V - V_f} \quad \text{and} \quad EP = \frac{K_e\,(V - V_f)}{V_f}$$

where V_f is the volume when under no strain and K_e is the bulk modulus. Substituting these values of OP and EP in (1)

$$\frac{1330 - 140V_e}{V_e} = \frac{K_e\,(V_e - V_f)}{V_f}$$

where V_e is the volume at equilibrium or

$$K_e = \frac{1330(1 - .105V_e)}{(V_e - V_f)} \frac{V_f}{V}$$

$$V_f = \frac{K_e V_e^2}{1330 + V_e(K_e - 140)}$$

$$V_e = \frac{(K_e - 140)V_f}{2\,K_e} \pm \sqrt{\frac{1330V_f}{K_e} + \left[\frac{(K_e - 140)V_f}{2\,K_e}\right]^2}$$

Ordinarily the total volume would be used in the calculation of the bulk modulus but since in the case of the osmotic pressure it is better to use the volume of water rather than the total volume, the calculation is simplified by using the same value in the bulk modulus calculation. The use of the total volume instead of the volume of water would simply result in a different value for the bulk modulus. It may be noted that the volume of dry gelatin, however, cannot be used, as was done in an earlier paper since in that case the formula would predict that the swelling would depend only on the concentration of the block, which is not the case. The determining factor is the concentration at the time the solution solidified and not the concentration at the time it happened to be put in the water. In other words, as with any elastic body, it is necessary to define the change in volume as the change from the volume when under no elastic strain.

TABLE I.

Swelling of Thin Layers of Gelatin on 2 mm. Glass Rods in H_2O at 5°.

V_f H_2O per gm gelatin when cast	V_e H_2O per gm gelatin after swelling		K_e	$\frac{K_e \times 77}{V_f}$
	Observed	Calculated $K_e = 500$		
2.35	3.5 ± .1	3.48	490	160
3.35	4.3 ± .2	4.40	550	126
5.0	5.7 ± .2	5.86	670	103
5.8	6.8 ± .3	6.56	350	46
Average.........			500	

As will be shown later this "elastic volume" is the actual volume when the solution solidified.

A number of experiments were made as previously described and the average value of K_e calculated. The results are given in Table I. K_e is expressed in mm. mercury pressure. The values of K_e vary somewhat but the equation is of such a form that the value of this constant is very sensitive to small differences in V_e. The value is really constant for the range of gelatin used as may be seen by the fact that the values of V_e calculated by assuming a constant value for K_e are equal, within the probable error of the measurement, to the observed values. If the value of K_e be defined as the force required to increase the volume by an amount equal to the volume of the dry gelatin in the block rather than the volume of water, then the value given must be multiplied by the ratio of the volume of dry gelatin to that of the water. Since 1 gm. of gelatin occupies about .77 cc., the pressure required to increase the block by an amount equal to the volume of the dry gelatin contained in it will be $K_e\ .77/V_f$. This value decreases in proportion to the concentration of gelatin, as would be expected. The same relation had been found by Sheppard (8) to hold approximately for the relation of modulus of elasticity to the concentration of the gelatin.

TABLE II.

Swelling of Gelatin in Various Forms.

$V_f = 5.8$

	On glass rods	On slides	Spheres	Thin sheets cut from cylinder
V_e	6.8	6.7	7.5	7.4
K_e	350	370	130	140
$\frac{K_e \times .77}{V_f}$	46	50	13	14

Since these experiments were made with gelatin on glass the gelatin is prevented from elongating by the glass rod. It might be expected therefore that gelatin alone would swell more. This is the case as shown by Table II which gives the equilibrium concentration for gelatin on slides, in the form of spheres or as thin pieces cut from a cylinder. It may also be noted that the value for the bulk modulus calculated on the basis of the volume of the dry gelatin agrees with that previously calculated indirectly from separate swelling and osmotic pressure experiments (9).

It has been assumed in these calculations that the gelatin when solidified is under no elastic strain and that this concentration is therefore the determining one for the swelling. As a corollary gelatin, the concentration of which has been changed after solidifying, is under elastic strain. It might be expected then that gelatin under these conditions would show the phenomenon of fatigue in common with other elastic bodies. That this is true is shown in Fig. 3. This experiment shows the result of keeping gelatin varying lengths of time at 20° after having been swollen or dried while solid. Curves 1 and 3 show that gelatin which has been allowed to solidify at 5° undergoes no change when kept in air at 20°. The swelling which occurs on replacing in water at 5° is the same whether or not the gelatin has been kept at 20°. Gelatin which has swollen at 5° before being placed at 20°, swells further on replacing at 5°, the amount of swelling depending upon the length of time the gelatin had been at 20°.

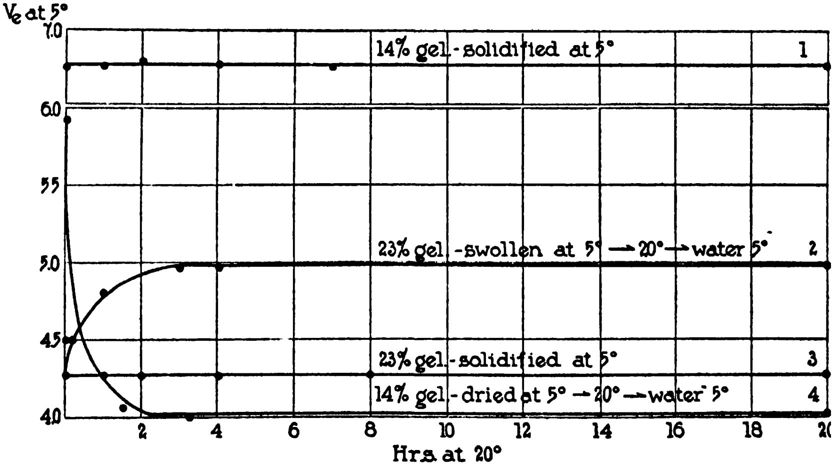

FIG. 3. Effect of time at 20° on subsequent swelling of gelatin at 5°. V_e = equilibrium volume reached on replacing in water at 5°.

After the first 2 hours at 20° further exposure to this temperature causes no further change in the behavior of the gelatin. The swelling now is the same as that of freshly solidified gelatin of the same concentration. Gelatin which has been partially dried shows the same behavior in that it swells much less after exposure to 20° and the swelling soon reaches a constant value. This loss of elasticity occurs very rapidly at 20° but is much slower at 5°. This is shown by Fig. 4. In this experiment, three series of rods coated with 30 per cent gelatin were allowed to swell to the equilibrium value at 5°. All but two were then removed and placed in air at 5°. They were then returned to the water after varying periods of time. In the meantime the two rods which had been left in water continued the slow secondary swelling. On returning the other rods to water they swelled rapidly, the more so the longer the time since the beginning of the experiment, and very rapidly approached the value of those that had been in the water continuously. This shows that the secondary swelling is due to the loss of elasticity and that it is continually going on. As stated before a large block will therefore not show any maximum value since the

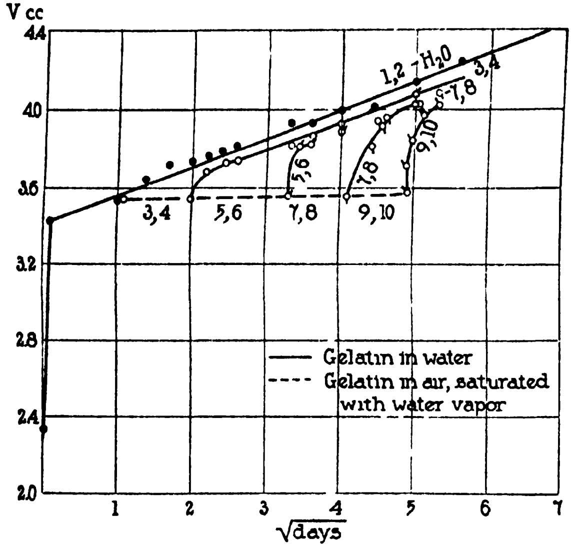

FIG. 4. Effect of time at 5° in air, after swelling, on subsequent swelling when the gelatin is replaced in water.

outside layers become fatigued and take in additional water before the internal layers have reached the equilibrium value. The same process will occur much more rapidly at a higher temperature so that even small blocks show no maximum. This secondary swelling will evidently continue until all the water has been taken up. If sufficient water is present a solution of gelatin will eventually result.

It may be noted that the secondary swelling in Fig. 4 is proportional to the square root of the time. The significance of this will be discussed under the kinetics of the process.

SUMMARY.

The swelling of isoelectric gelatin in water has been found to be in agreement with the following assumptions.

Gelatin consists of a network of insoluble material containing a solution of a more soluble substance. Water therefore enters owing to the osmotic pressure of the soluble material and thereby puts the network under elastic strain. The process continues until the elastic force is equal to the osmotic pressure. If the temperature is raised or the blocks of gelatin remain swollen over a period of time, the network loses its elasticity and more water enters. In large blocks this secondary swelling overlaps the initial process and so no maximum can be observed.

The swelling of small blocks or films of isoelectric gelatin containing from .14 to .4 gm. of dry gelatin per gm. of water is defined by the equation

$$V_e = \frac{(K_e - 140)}{2\,K_e} V_f \pm \sqrt{\frac{1330 V_f}{K_e} + \left[\frac{K_e - 140) V_f}{2\,K_e}\right]^2}$$

in which K_e = the bulk modulus $= \frac{PV_f}{(V_e - V_f)} = \frac{1330(1 - .105\,K_e)}{(V_e - V_f)} \frac{V_f}{V_e}$. V_e = gm. water per gm. gelatin at equilibrium; V_f = gm. water per gm. gelatin when the gelatin solidified.

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THE SWELLING OF ISOELECTRIC GELATIN IN WATER.

II. KINETICS.

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Measurements of the osmotic pressure and of the swelling of gelatin in salt solutions and of the swelling pressure of gelatin have led the writers[1] to assume that gelatin was a mixture of two substances or groups of substances, one of which is soluble and the other insoluble. Solid gelatin was therefore pictured as a network of the insoluble material holding a solution of the soluble protein in its meshes. The osmotic pressure of this soluble material was assumed to be the force which caused the block to swell. According to this mechanism the swelling of gelatin should be a special case of diffusion and should be fundamentally similar to osmosis. The peculiarity of swelling lies in the fact that the block of gelatin is both membrane and solution. Swelling differs from osmosis then in that the thickness of the membrane, *i.e.* the gelatin itself, increases during the course of the experiment, and the osmotic pressure is opposed by the elasticity of the gelatin rather than by the hydrostatic pressure. It might be expected therefore that the equation for the kinetics of the process while similar to that for osmosis would differ from it in some respects. This has been found to be the case.

As pointed out in the discussion of the kinetics of osmosis, the equation is of the same form whether the water is assumed to dissolve in and diffuse through the membrane or whether it is assumed to flow through capillary pores in the membrane. The only difference lies in the physical significance of the constants. There is some evidence that the flow of water through gelatin is also through pores and since this mechanism may be more easily analysed, it has been assumed in the present paper.

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[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161.

Formulation of the Equation.

According to Poiseuille's law the rate of flow of water through fine capillaries is proportional to the pressure times the fourth power of the radius and inversely proportional to the length of the capillary and the viscosity of the liquid. If the gelatin is assumed to consist of n pores per unit surface, then the total rate of flow of the water will be given by the expression

$$\frac{dv}{dt} = \frac{C'r^4nSP}{\eta h} \tag{1}$$

in which r is the radius of the pores, P the pressure, S the surface of the gelatin, h the length of the capillaries which is assumed to be the thickness of the gelatin, and η the viscosity of water. In order to use this expression the variable terms must be expressed as functions of v or t. In general, since the volume changes, the surface and thickness will also change. In the case of thin films of gelatin on glass, however, the surface may be assumed constant and the thickness therefore is equal to the volume divided by the surface, or $h = v/S$. The average distance traversed by the water in the case of swelling is half the thickness, so that $h/2$ must be used in place of h in equation (1).

Evaluation of P.—Since we are interested only in the amount of water which passes the outside surface of the gelatin it is not necessary to consider the pressure gradient in the gelatin, and the pressure may be assumed[2] equal to the average pressure. The pressure driving the water into the gelatin will be the difference between the swelling (osmotic) pressure which tends to cause the water to flow in and the elastic pressure of the gelatin which tends to force it out. Equilibrium is attained when these two pressures are equal. It was shown in the previous paper[3] that the swelling pressure at 5°C. from 0.14 to 0.4 gm. gelatin per gm. of water could be represented by the empirical formula

$$OP = \frac{1330 - 140V}{V},$$

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[2] The validity of this assumption is discussed in the preceding paper, Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 883.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 893.

where V is the volume of water per gm. of gelatin, and the elastic pressure by

$$EP = \frac{K_e(V - V_f)}{V_f},$$

where K_e is the bulk modulus and V_f is the volume when under no strain. Therefore

$$P = \frac{1330 - 140V}{V} - \frac{K_e(V - V_f)}{V_f}. \tag{2}$$

At equilibrium these two pressures are equal, *i.e.*

$$\frac{1330 - 140V_e}{V_e} = \frac{K_e(V_e - V_f)}{V_f} \quad \text{or} \quad K_e = \frac{1330(1 - .105V_e)\, V_f}{(V_e - V_f) \quad V_e}. \tag{3}$$

Evaluation of the Radius.—In the case of most membranes the radius of the pores would be constant, but in the case of gelatin this is probably not the case. If a block of gelatin is considered in any way analogous to a mass of separate fine particles of gelatin, then it is evident that the size of the pores which corresponds to the space between the particles will decrease rapidly as the particles swell. The exact function cannot be foretold as there is not sufficient evidence in regard to the actual structure of the gelatin. The simplest assumption, however, is that the radius of the pores between the particles would decrease as the radius of the particles increased or, approximately, $r^4 = c/V$. It will be assumed that the number of pores is constant. The size of the pores will also vary with the original concentration of the gelatin, but since this is constant for any one experiment it need not be taken into account here.

It is much more convenient to express the results in terms of the volume of water per gm. of gelatin rather than as the total volume. If g is the gm. of dry gelatin and V the volume of water per gm., then Vg equals the total volume of water, v, and $dv = gdV$.

As in the case of osmotic pressure, the equation may be solved in various ways depending upon which constants are used. The simplest expression is obtained in terms of K_e,[4] the bulk modulus, V_e the equilibrium volume, and V_f the volume when under no strain. In order to obtain the equation in this form equations (1) and (3) are added and the resulting equation solved for P.

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[4] Since V_e may be expressed in terms of K_e and V_f it would theoretically be possible to express the equation in terms of V_f and K_e alone. Actually, however, this can only be done if a number of swelling curves are made with similar

Substituting these values of h, P, r^4 and v in equation (1) and simplifying and collecting the constant terms, equation (1) becomes

$$\frac{dV}{dt} = \frac{2CS^2\,1330(bV + V_f)(V_e - V)}{V^3 g^2 V_e V_f},$$

where $b = K_eV_e/1330$; on integration, this becomes

$$C = \frac{g^2V_eV_f}{2 \times 1330\,S^2t}\left[\frac{V_o^2 - V^2}{2b} + \frac{(bV_e - V_f)(V_o - V)}{b^2} + \frac{2.3\,V_e^3}{(bV_e + V_f)} \log\left(\frac{V_e - V_o}{V_e - V}\right) - \frac{2.3\,V_f^3}{b^3(bV_e + V_f)} \log\left(\frac{V_f + bV}{V_f + bV_o}\right)\right] \qquad (4)$$

In these experiments the last term is negligible in most cases. If K_e, the bulk modulus, is small, however, the last term becomes significant. C is the rate of flow of water under unit pressure through unit thickness and unit area of gelatin of concentration V_f. In the units used in these experiments it is cc. per hour, per sq. cm. surface, per cm. thickness per mm. mercury pressure.

In order to test this equation a number of experiments were performed with films of gelatin of various concentrations on glass slides. The gelatin was heated to 40°C., the slides weighed and then dipped in the gelatin. They were then cooled and weighed again and placed at 5°C. in stoppered tubes with wet filter paper. It was shown in the preceding paper[3] that the pressure changes for the first 3 days and then remains constant, so that in order to obtain reproducible results and also since the pressure-concentration curve is based on the value of the equilibrium pressure, the slides were usually allowed to remain at this temperature for 3 days before immersing in water. They were

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films and the results averaged before applying the equation. This is due to the fact that the value of V_e, and hence of K_e, varies slightly in individual experiments and the value of C is very sensitive to small differences in the value of V_e, especially near the end of the curve. It is therefore necessary in the calculation of individual curves such as those given to use the value of K_e and V_e determined from the particular experiment in question.

TABLE I.

Swelling of Films of Gelatin on Glass.

Experiment	S	g	V_f	V_e	t	V	$C \times 10^5$
	sq. cm.	*gm.*	*cc.*	*cc.*	*hrs.*	*cc.*	
2/2 *a*	25	.05	6.1	6.9	0	6.12	
					.05	6.50	1.15
					.10	6.65	1.00
					.20	6.77	.80
1/13 *a*	25	.045	6.0	6.75	0	6.0	
					.05	6.32	.63
					.10	6.48	.61
					.20	6.66	.49
2/2 *b*	25	.12	6.1	6.9	0	6.12	
					.32	6.50	1.05
					.55	6.65	1.03
					1.00	6.75	.85
c	25	.14	6.0	6.75	0	6.0	
					.2	6.27	1.22
					.4	6.45	1.30
					.8	6.62	1.30
1/24 *a*	25	.063	5.9	6.75	0	2.45	
					.10	5.0	.40
					.20	5.82	.48
					.40	6.42	.56
1/20	27	.16	5.0	5.4	0	5.0	
					.5	5.15	.140
					1.0	5.225	.123
					1.5	5.275	.120
1/24 *b*	27	.052	3.80	5.0	0	1.55	
					.05	3.3	.120
					.10	4.0	.118
					.20	4.45	.119
1/13 *b*	25	.10	3.35	4.20	0	3.35	
					.05	3.70	.360
					.10	3.82	.284
					.20	3.95	.235
12/29	42	.31	2.35	3.4	0	2.23	
					.125	2.62	.125
					.25	2.82	.122
					.50	3.0	.110

then weighed again and placed in water at 5°C. They were removed at intervals, the excess water removed with soft filter paper and weighed in air at the same temperature. This is necessary since even slight warming destroys the elasticity and greatly increases the swelling. It is also important to be sure that no change in weight has occurred before the slides and gelatin are weighed for the first time, since in the calculations it is assumed that the gelatin is of the same concentration as the solution of gelatin in which they were dipped. In some experiments the gelatin was allowed to dry partially at 5°

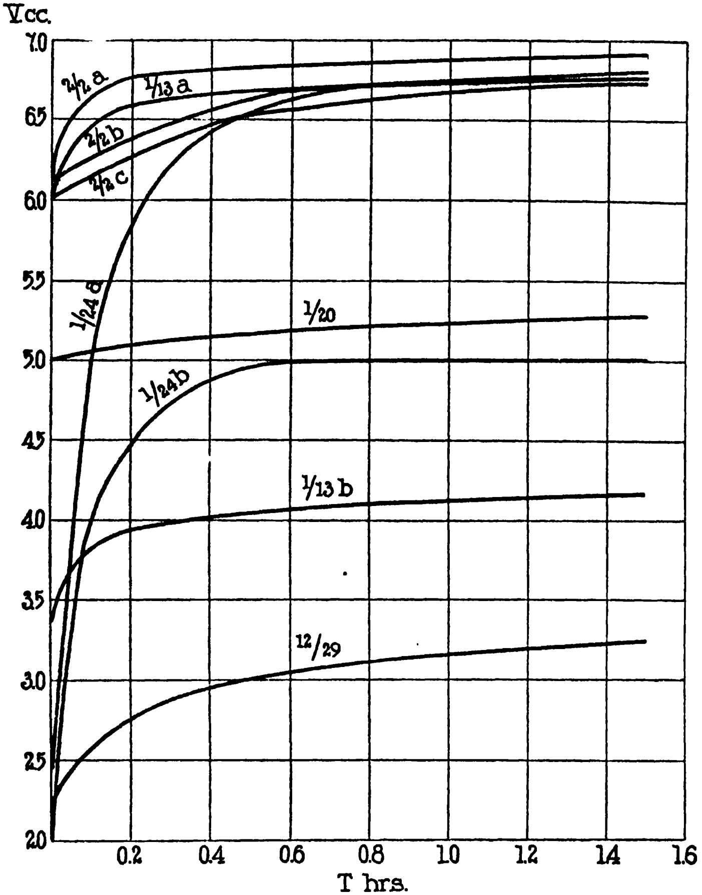

FIG. 1. Swelling films of gelatin on glass.

before placing in water. In this case the gelatin swells much more than the same concentration of gelatin which had been solidified at that concentration. It was shown in the preceding paper[3] that the equilibrium reached could be calculated by assuming that the concentration when under no strain, V_f, was the concentration when the gelatin solidified. That is, the gelatin will swell to the same final value as though it had not been dried. This is strictly true only within certain limits and provided too long a time has not elapsed after drying,

TABLE II.

Swelling of Gelatin Spheres and Plates.

13.9 per cent gelatin heated to 40° and allowed to drop slowly into 200 cc. cold toluene in 250 cc. graduate. 20 spheres used for experiment.

13.9 per cent gelatin solidified in test-tubes. 5°, 3 days. Gelatin removed by warming and sections cut.

Spheres					
g	V_f	V_e	t	V	$C \times 10^6$
gm./sphere	*cc.*	*cc.*	*hrs.*	*cc.*	
.002	6.4	7.7	0	6.4	
			1.0	6.8	.85
			2.0	7.06	.84
			4.0	7.40	.92
.005	6.4	7.7	0	6.4	
			1.5	6.8	1.02
			2.8	7.06	1.10
			7.0	7.40	0.97
Sections cut from cylinder 2.0 cm. diameter					
.037	6.3	7.5	0	6.2	
			.5	6.9	2.00
			1.0	7.13	1.75
			2.0	7.35	1.65
.10	6.3	7.5	0	6.2	
			3.0	6.9	2.45
			5.75	7.13	2.20
			11.0	7.35	2.15

as otherwise the gelatin becomes fatigued and V_f assumes a different value.

The results of some of these experiments are shown in Table I and in Fig. 1. The value of C varies slightly, but as a whole the equation appears to fit the experiment in a satisfactory way. The value of C is greater the greater the value of V_f, that is the more dilute the gelatin. This means that water flows more easily through dilute gelatin, which is a reasonable result. It can be confirmed, as will be shown below, by direct measurement. In spite of the precautions noted some of the experiments show an anomalous course in that the gelatin swells either more or less than others of the same series. Experiment 1/20 is an example. The swelling in this case was very much less than would be expected from the average bulk modulus, and therefore the value of C although reasonably constant for this one experiment does not fit in with the other experiments. If the gelatin is placed in water immediately after solidifying, the opposite result is obtained, the gelatin swelling much more. This was ascribed to the fact that the pres-

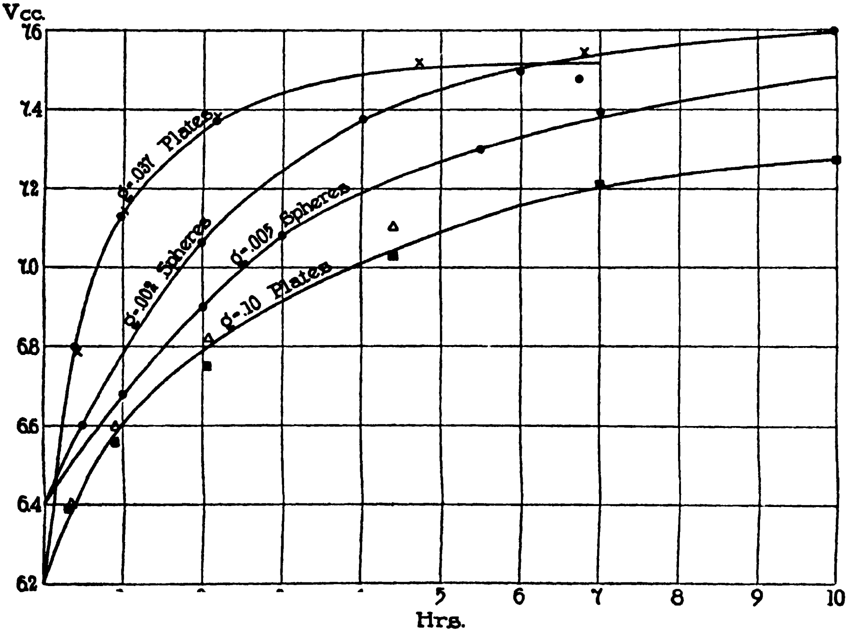

FIG. 2. Swelling of gelatin plates and spheres.

sure is too high since it is assumed that the block is a saturated solution of one of the constituents and it requires time for the equilibrium concentration to be reached. In such cases also it was found that the value of C would be constant for that particular experiment but would differ from the other values.

Plates of Gelatin.—In the case of thin plates cut transversely from a cylinder of gelatin conditions are approximately the same as in the case just considered, since here also the greatest change in dimension is an increase in thickness and the surface may be considered constant. The results of some experiments with such plates are shown in Fig. 2 and Table II. The results have been calculated by formula (4) as before.

Spheres.—In the case of spheres the thickness instead of increasing directly as the volume increases only as the cube root of this value. The average distance which the water has to move is 1/6 the radius, which is 1/2 the ratio of volume to surface or

$$h/2 = r/6.$$

In these experiments the volume increases less than 20 per cent, so that the cube root of the volume may be considered constant for any one sphere and h assumed equal to $r_o/3$. This assumption is made in order to avoid the mathematical difficulties attendant on the integration of the fractional power of V. The surface also increases, but since the significant factor is the total number of pores and since the number presumably remains the same during any one experiment, S will also be considered a constant for any one experiment. In any case the effect of considering S variable would be within the limit of experimental error. Therefore $S = 4\pi r_o^2$ and $S/h = 12\pi r_o$, or $24\sqrt[3]{v_o} = 24\sqrt[3]{V_o g}$, approximately.

Substituting this value for S/h, equation (1) becomes

$$\frac{dV}{dt} = \frac{2 \times 1330 \times 24C\sqrt[3]{V_o g} \times (bV + V_f)(V_e - V)}{g\, V^2 V_e\, V_f};$$

or on integration:

$$C = \frac{g^{\frac{2}{3}}\, V_f V_e}{2 \times 1330 \times 24\sqrt[3]{V_o}\, t}\left[\frac{V_o - V}{b} + \frac{2.3V_e^2}{bV_e + V_f}\log\frac{V_e - V_o}{V_e - V} + \frac{2.3V_f^2}{b^2(bV_e + V_f)}\log\frac{bV + V_f}{bV_o + V_f}\right] \tag{5}$$

The last term is again negligible except when K_e, the bulk modulus, is small, *i.e.* when b is small.

TABLE III.

Swelling of Gelatin Cylinders.

Linen thread 15 cm. long dipped into melted gelatin.

$l = 15$ cm.

Experiment	s	V_f	V_e	t	V	$C \times 10^{8}$
	gm.	*cc.*	*cc.*	*hrs.*	*cc.*	
11/1	.08	5.80	6.90	0	5.8	
				.5	6.15	1.16
				1.0	6.37	1.15
				2.0	6.67	1.35
	.15	5.80	6.90	0	5.8	
				1.0	6.17	1.23
				2.0	6.40	1.22
				4.0	6.60	1.05
10/7	.25	6.0	7.8	0	6.0	
				2	6.5	1.40
				6	6.97	1.22
				16	7.50	1.18
11/2	.09	3.35	4.30	0	3.30	
				1.0	3.72	.170
				2.0	3.92	.162
				4.0	4.13	.154
11/2	.12	2.35	3.75	0	2.10	
				1	2.80	.150
				2	3.10	.145
				4	3.45	.145
10/13	1.0	2.35	4.5	0	2.35	
				10	3.20	.155
				20	3.60	.155
				40	4.00	.145

The results of the experiments are shown in Table II and Fig. 2. The values of C are again as constant as could be expected.

Cylinders.—The cylinders of gelatin were made by the repeated dipping of a thread in liquid gelatin so that the gelatin on swelling did not increase in length but only in diameter. The average distance traversed by the water will be in this case $r/4$ and the total pore number Sn will equal $2\pi r_0 l$, where l is the length of the cylinder. In order to avoid fractional exponents it will be assumed again that $S = 2\ \pi r l$, and therefore $S/h = 4\pi\ l$. Substituting this value, equation (1) becomes

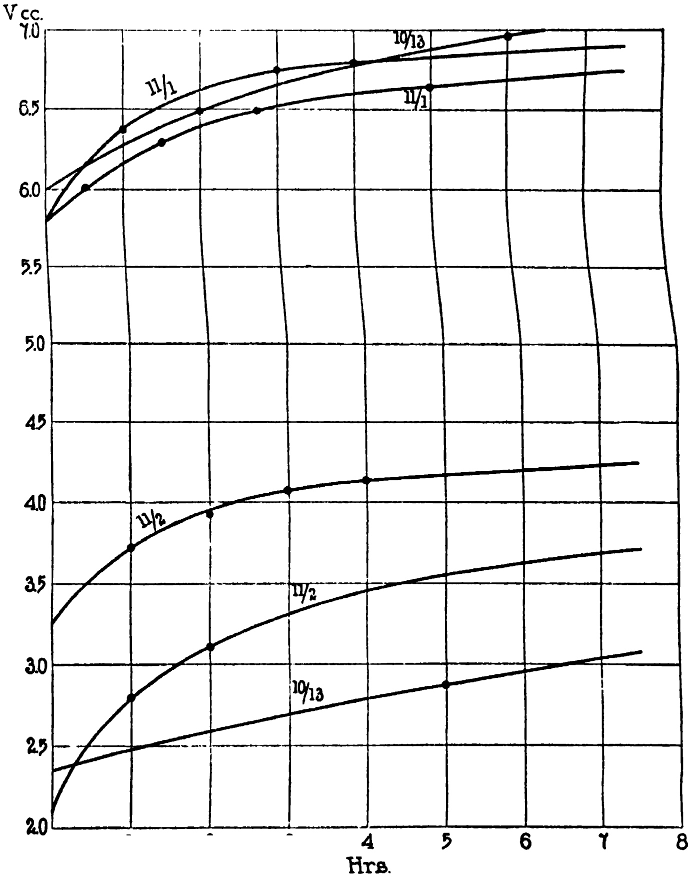

FIG. 3. Swelling of gelatin cylinders.

$$\frac{dV}{dt} = \frac{2 \times 1330 \times C \times 4\ \pi\ l(bV + V_f)\,(V_e - V)}{Vg\ \ VV_fV_e}.$$

Integrating and collecting constant terms the equation for the rate of swelling of cylinders is therefore:

$$C = \frac{gV_fV_e}{2 \times 1330 \times 4\,\pi\, ll}\left(\frac{V_o - V}{b} + \frac{2.3V_e^2}{bV_e + V_f}\log\frac{V_e - V_o}{V_e - V} + \frac{2.3V_f^2}{b^2\,(bV_e + V_f)}\log\frac{V_f + bV}{V_f + bV_o}\right)$$

The effect of swelling on the rate, owing to the simplifying assumptions used, is the same as for spheres but the effect of varying the initial size is different. The results of the experiment with cylinders are given in Table III and Fig. 3.

Direct Measurement of C.—According to the derivation of the equations, C should be the rate of flow of water in cc. per hour through a cylinder of gelatin having 1 cm. cross-sectional area, 1 cm. long, under a pressure of 1 mm. mercury. This value can be determined directly by measuring the flow of water through gelatin. Gelatin was allowed to solidify in glass tubes of 0.5 cm. diameter so as to form a plug 1 cm. long.[5] Water was then forced through these plugs under 20 cm. mercury pressure and the amount passing through measured in a pipette calibrated in 0.001 cc. The measurement was made at 5°C. Since when gelatin swells there is a loss of volume of the system as a whole, it is necessary to correct the observed rate for the change in volume when under no pressure. In the case of 14 per cent gelatin ($V_f = 6$), this correction is negligible; in the case of 23 per cent gelatin it is significant; and in the case of 30 per cent gelatin it is so large as to render the measurement uncertain. The values for the more dilute gelatin agree as well as could be expected with the value calculated from the rate of swelling measurement and show about the same effect of the original concentration. The plugs were then placed in water at

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[5] It may be noted in this connection that the membrane cannot be held in place by a rigid support since in that case the pressure at first presses water out of the membrane. This continues until the osmotic pressure of the membrane itself is equal to the applied pressure. In other words, no matter what concentration of gelatin is used to make the membrane, water will be removed or taken up so that when a steady filtration rate is reached the concentration of gelatin in the membrane is that which will give an osmotic pressure equal to the applied pressure.

5° for 24 hours and the measurement repeated. The rate of flow was now so slow as to be impossible to measure with any accuracy, thus verifying directly the assumption made in the beginning that the permeability decreased with the increase in swelling. In the case of the direct measurement the gelatin was prevented from expanding by the glass tube so that it is not surprising that the effect of swelling is much more marked than in the case of blocks not enclosed in a solid wall. A summary of the values of *C* is shown in Table IV.

The results as a whole show that the equations fit the time rate curves quite well. Owing to the number of constants and the uncertainty of the exact value for the equilibrium volume, this agreement might be regarded as accidental. What is much more significant, in the writer's opinion, is the fact that the equations lead to a value for the rate of diffusion of water through gelatin that has been checked by direct determination and also that they express correctly the effect on the rate of swelling of varying the initial size or shape of the block. It may be noted for instance that in the case of thin films the equation

TABLE IV.

Summary Value of C.

$C \times 10^5$ = cc. per mm. Hg pressure, per hour, per sq. cm. surface per cm. thickness.

Concentration gelatin V_f	Cylinders	Plates	Spheres	Film on glass	Average	*C* by direct determination	
						Not swollen	Swollen
6.0	1.15	1.7	.9	(.13)			
	1.20	2.2	1.0	1.0			
	1.30			1.0			
				1.3	1.1	1.0	<.10
				.60			
				.50			
3.35	.16			.30	.2	.5	<.10
				.12			
2.35	.15						
	.15			.12	.13		

predicts that the rate of swelling per gm. of dry gelatin decreases inversely as the square of the weight of dry gelatin, in the case of spheres it decreases approximately inversely to the 2/3 power of the weight of dry gelatin, while in the case of cylinders the rate is inversely proportional to the first power of the weight of dry gelatin. If the rate of swelling is expressed simply as the total amount of water taken in per unit of time, then the equation predicts that in the case of thin films the rate is inversely proportional to the size of the block, or if the surface is constant, to the thickness. This is the result obtained in all work on swelling.

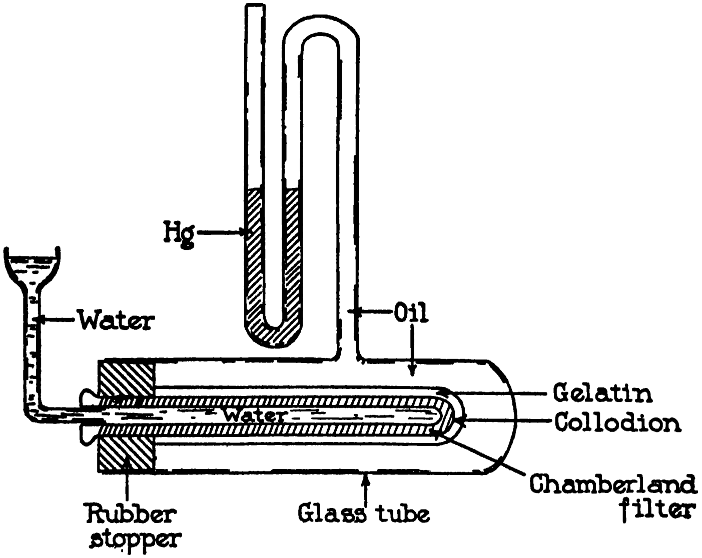

FIG. 4. Apparatus for measurement of swelling of gelatin on collodion-coated thimble.

Swelling under Experimental Conditions Which Avoid the Complicating Factors.

The preceding experiments show that the mechanism assumed for the swelling of gelatin predicts the results with considerable accuracy, but they are open to the objection that the formulas contain a number of constants and the agreement may therefore be accidental. If the mechanism is correct, however, it should be possible to predict conditions under which the various secondary complications disappear or become negligible. These complications are due to the fact that the swelling pressure is opposed by the elasticity of the gelatin, a value which cannot be determined directly; and secondly to the fact that the resistance of the gelatin to the passage of the water is constantly increasing, due partly to the increase in the thickness of the layer of gelatin and partly to the decrease in the size of the pores. It was shown in the preceding paper[3] that at higher temperatures the elasticity is rapidly destroyed. It was also found that the resistance offered by collodion is very much greater than that offered by gelatin. If therefore a thin film of gelatin is coated on collodion so that the water has to pass through the collodion, the resistance offered by the gelatin will be a negligible part of the total resistance. If the experiment is carried out at 25° the elasticity of the gelatin will be partially destroyed and the term representing this force will also disappear. These conditions can be fulfilled by the apparatus shown in Fig. 4. The Chamberland filter was coated with collodion and then with gelatin. The gelatin was left at 5° for 24 hours in air and the apparatus set up as shown in a constant temperature bath at 25°C.

Under these conditions all the terms in equation (1) are constant except the pressure. The pressure will be equal to the swelling pressure minus the hydrostatic pressure. The curve for the swelling pressure of gelatin at 25° may be nearly superimposed on the curve at 5° by reducing the concentration of gelatin. The gelatin used had the same swelling pressure at 25° as 23 per cent gelatin at 5°, so that the same formula will apply for the pressure as was used for the experiments at 5° provided the concentration of gelatin be assumed to be 23 per cent. The swelling pressure is now opposed by the hydrostatic pressure just as in the osmotic pressure experiments, and the total pressure may therefore be written

$$P = OP - HP = \frac{1330 - 140V}{V} - M - Kg(V - V_o),$$

where M is the initial hydrostatic pressure and K is the mm. pressure per cc. increase in volume. Substituting this value of P, equation (1) becomes

$$\frac{dV}{dt} = \frac{CSnr^4}{h\eta g}\left[\frac{1330 - 140V}{V} - M - Kg(V - V_o)\right];$$

and bringing all the constant terms together and integrating:

$$C = \frac{2.3 g V_e}{(1 + bV_e)\,1330\,St}\left(V_e \log \frac{V_e - V_0}{V_e - V} - \frac{1}{b} \log \frac{1 + bV}{1 + bV_0}\right)$$

in which $b = KgV_e/1330$.

C in this case should be the cc. of water passing through 1 sq. cm. of the collodion-coated thimble per hour per mm. mercury pressure. When the swelling experiment was concluded the gelatin was removed and this value of C was determined directly. The results of this experiment are given in Table V and Fig. 5. The difference in the values of C for the two experiments is presumably due to differences in the membranes. The value for the second experiment is quite close to the figure determined directly. At this temperature and under these conditions the final amount of swelling is not determined by the concentration of the gelatin but depends only on the hydrostatic

TABLE V.

Swelling Gelatin on Collodion-Coated Thimble at 25°.

Experiment I. $g = 2.5$ $K = 220$ $V_e = 3.68$ $S = 103$

t	V	$C \times 10^4$
hrs.		
0	3.35	
.2	3.42	.45
.4	3.49	.51
.6	3.55	.57

Experiment II. $g = 2.0$ $K = 24$ $V_e = 5.3$ $S = 103$

t	V	$C \times 10^4$
hrs.		
0	3.35	
.4	3.78	1.05
.8	4.10	1.02
1.6	4.52	1.02
3.2	5.0	1.06

By direct determination..1.2

pressure. This confirms the assumption made above that at 25° the elasticity of the gelatin does not enter into the equation.

Secondary Swelling.

Under the conditions adhered to in these experiments an apparent maximum is rapidly reached. This value has been called the equilibrium volume. If measurements are carried on over a long period of time, however, it will be found that there is a slow steady increase in volume. This is shown in Fig. 6, in which the swelling has been plotted against the square root of the time. This secondary swelling has been ascribed to the fatigue of the elastic force of the gelatin, and evidence was submitted in the preceding paper[3] to show that this was really the case. Fig. 6 shows that the rate of this secondary swelling is independent of the size of the block while the primary swelling is inversely proportional to the square of this quantity. This also bears out the idea that the secondary swelling is due to the fatigue of the gelatin, and hence is not regulated by the rate of diffusion of the water. This fatigue effect may be ascribed to a change in the value of V_f, which tends to approach the actual volume. It is possible to gain some idea

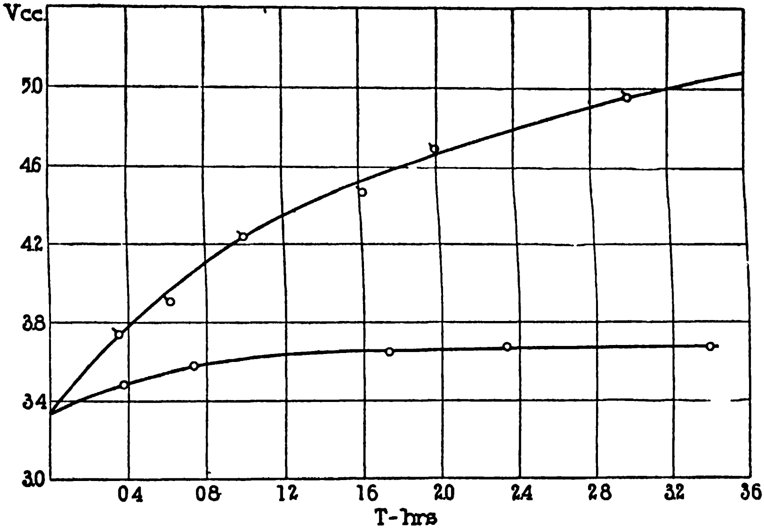

FIG. 5. Rate of swelling of gelatin on collodion-coated thimble

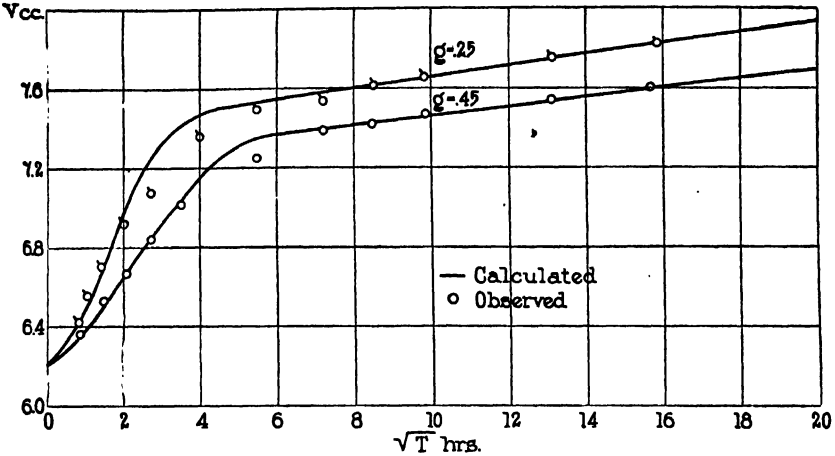

FIG. 6. Complete swelling curve of 13.5 per cent gelatin.

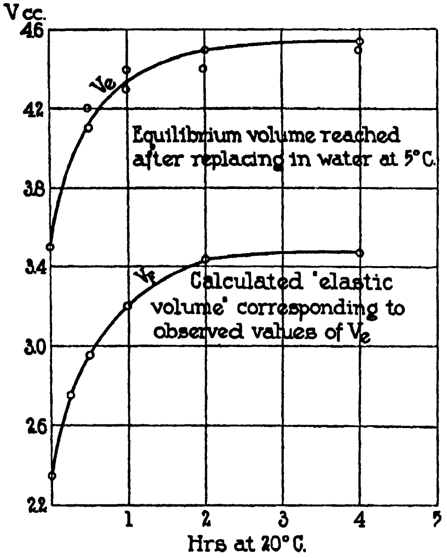

FIG. 7. Effect of time at 20° on subsequent swelling of swollen films of 30 per cent gelatin.

of the rate of change of this value by allowing swollen gelatin to remain varying lengths of time at 20° in air and then noting the swelling on returning the gelatin to water at 5°. The final volume reached in water gives the value of V_e, and if the bulk modulus is constant the value of V_f at any time can be calculated. When these values of V_f are plotted against the time the block was kept in air, the second curve given in Fig. 7 is obtained. This curve is approximately logarithmic as might be expected and shows that the change of V_f with time is proportional to the difference between its value at time t and the equilibrium value V_{f_e}. Or

$$\frac{dV_f}{dt} = C(V_{f_e} - V_f),$$

which on integration becomes

$$C = \frac{1}{t} \log \frac{V_{f_e} - V_{f_0}}{V_{f_e} - V_f}.$$

Table VI shows that this equation holds at least as an approximation.

In this experiment the block was removed from so water that there was no change in the actual volume during the time V_f was changing, *i.e.* V_{f_e} was constant. In the actual experiments however, as soon as V_f changes V_{f_e} also increases. $V_{f_e} - V_f$ may therefore be considered as approximately constant, or

TABLE VI.

Change of V_f with Time.

V_f-t		
t	V_f	K
hrs.		
0	2.35	
.25	2.74	.72
.50	2.98	.72
1.0	3.20	.62
4.0	3.47	

$$\frac{dV_f}{dt} = C', \text{ and } V_f = C't + \text{a constant.}$$

It was shown in the previous paper[3] that the equilibrium volume V_e was related to V_f by the equation $V_f = KV_e^2/1330 + V_e\,(K - 140)$.

The denominator of this equation may be considered constant for moderate changes in the value of V_e and the equation written

$$V_e^2 = C''V_f = C'''t; \tag{6}$$

or, since under these conditions V_e is the actual volume, $V = C\sqrt{t}$. This is the result shown in Fig. 6.

Complete Formula for Swelling.

Since the formula for the primary swelling has already been given the sum of this formula and formula (6) above will evidently represent the entire process. The equation for the primary swelling is too complicated to handle conveniently in this way, and it is necessary to omit some of the complicating factors. Since the primary swelling curve is basically logarithmic it is to be expected that it would fit the ordinary monomolecular formula provided the proper value of V_e is chosen. This of course deprives the formula of any theoretical meaning since V_e is actually determined by experiment, but may serve to give an expression for the first part of the primary swelling curve which can be used. This turns out to be the case, and it also happens that the value of V at which the secondary square root curve cuts the V axis may be used. The first part of the primary swelling may be represented therefore by the equation

$$Ct = \log \frac{V_e - V_o}{V_e - V''} \tag{7}$$

where V' is the amount of swelling due to the primary process plus the original volume, or $V' = V_e - 10^{A-Ct}$; and the secondary swelling as

$$V'' - V_e = C\sqrt{t}, \tag{8}$$

where V_e in (7) is taken arbitrarily as the value of V_e in (8) when $t = 0$.

Since V' in equation (6) is the original volume plus the increase in volume due to the primary swelling, and $V'' - V_e$ is the increase in volume due to the secondary swelling, the total volume at any time will be the sum of these quantities or

$$V = C\sqrt{t} + V_e - 10^{A - C't}.$$

The solid lines in Fig. 6 were calculated by means of this formula and follow the general course of the actual experiment. In some of the experiments the fit was much better but there is naturally always a discrepancy near the end of the primary swelling curve.

It follows from the mechanism outlined above that if a large block is used, especially at a higher temperature, the primary swelling should be completely overshadowed by the secondary. That is, in a large block the outside layers will become fatigued and take in more water before the inside layers have swollen at all. The entire course of the swelling should then be represented by the square root curve. The increase in weight of a block of 30 per cent gelatin containing about 35 cc., at 27°, is shown in Fig. 8. The process evidently follows the square root curve quite closely. According to this mechanism the gelatin should increase indefinitely or until it had taken up all the available water, and this is known to be the case.

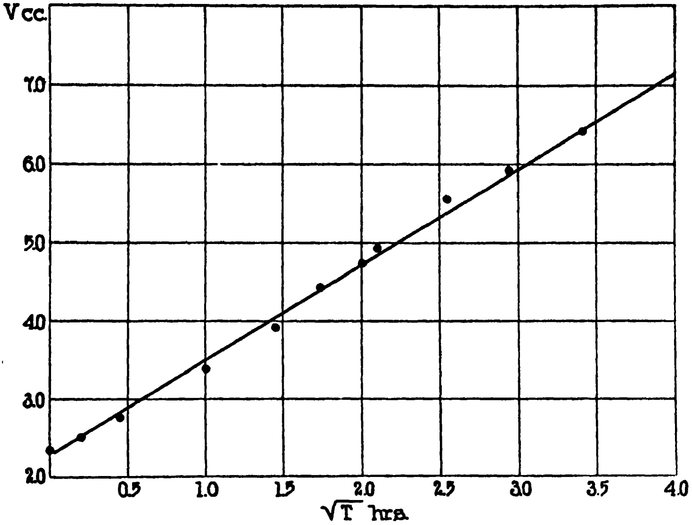

FIG. 8. Swelling of large block, 30 per cent gelatin at 27°. $g = 10$.

Application to Other Types of Swelling.

If the theory of the kinetics of swelling outlined in this paper is correct it should apply in a general way to any system in which only the solvent enters the material undergoing swelling, or in which the swelling is not affected by other substances present in solution. In the case of swelling due to a Donnan equilibrium, the pressure in the particle depends on the distribution of an electrolyte as well as on the entrance of the solvent. The theory would only apply to such systems provided conditions were such that the rate was determined by the passage of water into the solid. This condition is probably rarely if ever realized.

SUMMARY.

It has been assumed that gelatin consists of a network of an insoluble material enclosing a solution of a more soluble material.

The swelling of gelatin is therefore primarily an osmotic phenomena in that the water tends to diffuse in owing to the osmotic pressure of the soluble material. This osmotic pressure is opposed by the elasticity of the insoluble constituent, and equilibrium results when these two pressures are equal.

The rate of the entrance of water should then obey Poiseuille's law, provided the variable terms are expressed as functions of the volume. Equations have been derived in this way which agree quite well with the experimental curves and which predict the proper relation between the size and shape of the block and the rate of swelling. They lead to a value for the rate of flow of water through gelatin which has been checked by direct measurement.

The mechanism assumed predicts that at a higher temperature and under conditions such that the water has to pass through collodion before reaching the gelatin, the experiment should follow the same course as that of osmosis discussed previously. This was also found to be the case.

The slow secondary increase in swelling is ascribed to fatigue of the elastic properties of the gelatin. The rate of this secondary swelling should therefore be independent of the size of the block, in contrast to the rate of primary swelling which is inversely proportional to the size. It can further be shown that this secondary swelling should be proportional to the square root of the time, and also that with large blocks at higher temperatures the entire swelling should be of this secondary type. These predictions have also been found to be true.

ON THE NATURE OF THE DYE PENETRATING THE VACUOLE OF VALONIA FROM SOLUTIONS OF METHYLENE BLUE.

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I.

INTRODUCTION.

Experimental results[1] favor the theory that a basic dye penetrates a living cell very rapidly in the form of free base (which predominates at a high pH value) but so slowly in the form of salt (which predominates at low pH values) that its penetration is comparatively negligible. If this theory were correct, we should not expect a dye like methylene blue, which is very strongly basic,[2] to enter a living cell, since at the range of pH values generally available for living cells this dye exists in the form of salts. Yet methylene blue is widely known as one of the most commonly used vital stains. What is the explanation for the discrepancy between the theory presented and the observed facts? Does this indicate that the theory is inadequate, or does it mean that the dye which penetrates is not methylene blue but a less basic lower homologue, such as azure B or trimethyl thionine,[3] which is found in methylene blue solutions especially at a higher pH value and which is capable of existing partly in form of free base? It has been stated by some investigators[4] that lower homologues are found in samples of methylene blue, and others[5] have found that in presence of air and with an alkaline reaction methylene blue in aqueous solution is partly converted to methylene

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[1] Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350. Irwin, M., *J. Gen. Physiol.*, 1925–27, viii, 147; 1925–26, ix, 561; 1926–27, x, 75.

[2] For discussion of the apparent dissociation constant of methylene blue *cf.* Clark, W. M., and his collaborators (Clark, W. M., Cohen, B., and Gibbs, H. D., *Pub. Health Rep., U. S. P. H., No. 23*, 1925, 1131).

[3] The apparent dissociation constant of azure B has not been determined, but we have the following reason to assume that it is a weaker base than methylene blue. In general it is found that a substance whose amino groups are completely substituted with alkyl radicles, such as tetramethyl ammonium hydroxide,

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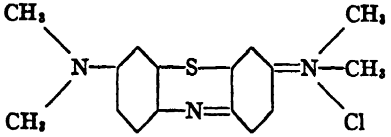
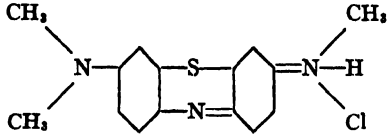
is a strong base; while a substance whose amino groups are only partially substituted by alkyl radicles, such as trimethyl ammonium hydroxide is a weaker base. Since amino groups of methylene blue or tetramethyl thionine are completely substituted with alkyl radicles, while those of azure B or trimethyl thionine are only partially substituted by alkyl radicles, as shown below, it would seem reasonable to suppose that methylene blue behaves like a strong base while azure B behaves like a weaker base.

Methylene blue or tetramethyl thionine.



Azure B or trimethyl thionine.



Difference between the chemical structure of the dye in form of free base and that of the dye in form of salt must be left undecided until further studies are made. It is uncertain as to whether such a difference between free base and salt as represented in the previous publication (Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 76) is correct. It may very well be that the salt is represented by the structure given above and that the free base is represented by a structure in which the Cl is replaced by the OH, or it is an anhydro-base.

[4] Scott, R. E., and French, R. W., *Milit. Surg.*, 1924, lv, 1. Conn, H. J., Biological stains, 1925, published by the Commission on Standardization of Biological Stains. Haynes, R., *Stain Technol.*, 1927, ii, 8. A delicate and reliable method for the detection of the presence of trimethyl thionine in methylene blue has been devised by Holmes and will appear in an early issue of *Stain Technology*. By means of this method it was found that the purest samples of methylene blue available for testing invariably contained small proportions of trimethyl thionine.

[5] Bernthsen, A., *Ann. Chem.*, 1885, ccxxx, 137. Kehrmann, F., *Ber. chem. Ges.*, 1906, xxxix, 1403. Baudisch, O., and Unna, P. G., *Dermat. Woch.*, 1919, lxviii, 4.

azure, which was found[6] to enter chloroform and to appear red. Furthermore, Kehrmann[7] has stated that methylene azure, which is a mixture of trimethyl thionine (azure B) and asymmetrical dimethyl thionine (azure A), enters substances like ether, chloroform, and benzene in form of a base and not in form of a salt, while methylene blue is not soluble in ether.

In view of the fact that the dye from methylene blue solution does not enter[8] the living cells except when the pH value of the solution is high, we may have a good reason for suspecting that the dye capable of entering a living cell is not methylene blue (in the form of a salt), but a less basic homologue (in the form of a free base), just as in the case of absorption by a substance like ether (already discussed). In this case the theory first presented would prove adequate.

One way to test this question is to study by spectrophotometric analysis the nature of the dye inside and outside the living cell. Heretofore this has not been attempted. The writer therefore proposes to give in the present paper[9] a series of spectrophotometric analyses of the dye penetrating from solutions of methylene blue into the vacuole of the living cell of marine alga *Valonia macrophysa*.

II.

Penetration of Dye into Valonia from a Solution of Methylene Blue.

Details of technique will be omitted here since they have previously been given by the writer.[10] Mention may, however, be made of several points of importance. Medium sized cells were chosen to avoid errors caused either by injury or by contamination of the sap from the stained cell wall. If too large a cell was employed, it took so long for the dye to collect in the vacuole that injury often occurred before there was a sufficient quantity of dye in the sap for spectrophotometric analysis. If too small a cell was used, the dye derived from the stained cell wall when the cell was punctured by a capillary tube for the purpose of collecting the sap exceeded the concentration of dye in the vacuole, which had penetrated from the external dye solution before the cell was removed from the solution and punctured.

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[6] *Cf.* Baudisch, O., and Unna, P. G., Foot-note 5.

[7] *Cf.* Kehrmann, Foot-note 5.

[8] Harvey, E. N., MacArthur, J. W., and Irwin, M., see Foot-note 1.

[9] A preliminary report of these analyses has been made by the writer (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 425).

[10] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 271.

It is desirable to possess a sound basis for judging the condition of cells during the experiment. It is not a difficult matter to determine an irreversible injury, but a reversible injury is almost impossible to detect. It was therefore necessary to have a more or less arbitrary basis for judging if the cells were injured. Cells during experiment were considered to be uninjured (1) if they continued to live after they were kept in the test solution for several hours beyond the time required for experiment, (2) if they were found to be living a day or so after they had been transferred from the test solution to normal sea water, (3) if the turgidity of the cell, as detected by touch, remained the same as that of control cells (as the cells become injured they lose their turgidity).

The pH values of the sea water employed were about pH 5.5, 9.5, and 10.9. The pH value of the Bermuda sea water in which the dye was dissolved was altered and determined in the following manner. To sea water, hydrochloric acid was added until the color of the test-tube containing the sea water and brom cresol purple matched that of the standard phosphate buffer solution at pH 6 containing the same concentration of the indicator and 0 6 M sodium chloride, which roughly corresponds to the halide concentration of Bermuda[11] sea water. To sea water sodium hydroxide was added until the color of the test-tube containing the sea water and cresol phthalein matched that of the test-tube containing standard borate buffer solution at pH 9.7 or at pH 11.2, containing the same concentration of the indicator and 0 6 M sodium chloride. Owing to the slight difference in the pH value of different samples of Bermuda sea water the volume of hydrochloric acid and sodium hydroxide added varied slightly, but to 100 cc. of sea water on an average was added 1 08 cc. of 0.2 M hydrochloric acid (for pH 5.5) or 0.8 cc. of 0.2 M sodium hydroxide (for pH 9.5) or 0 45 cc. of 0.5 M sodium hydroxide (for pH 10.9).

Since the addition of sodium chloride alters the pH values of the buffer solutions, the pH values of the standard phosphate and borate buffer solutions containing 0.6 M sodium chloride were determined by means of the hydrogen electrode, and they were found to be pH 5.5 (phosphate), pH 9.5 (borate), and pH 10.9 (borate). These pH values represent only approximate pH values of the given sea water, because the colorimetric determination is not accurate for the following reason. Since in the standard buffer solutions the halide content of the sea water is represented only by sodium chloride, and since some salts are known to change the color of the indicators more than the others, the pH value of the standard buffer solution containing 0.6 M sodium chloride and that of the sea water may not be exactly the same even though the color of the test-tube containing the one matches that of the test-tube containing the other.

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[11] Bermuda sea water contains about 0.58 M halides (Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, vii, 637).

Furthermore, above pH 10.3, the magnesium in the sea water is precipitated, which in some cases interferes with colorimetric determination, and no indicators are very sensitive in this range, so that at pH 10.9 the error may not be small. But no effort was made to avoid such sources of error since only the relative pH values were desired for these experiments. These pH values therefore are sufficiently accurate to serve the purpose in the present case, though they are approximate values.

Methylene blue is partially salted out in sea water so that two kinds of solutions were used; (1) a dye solution in which all the precipitate was allowed to remain, (2) a dye solution from which the precipitate was in greater part removed by filtering. In both cases the concentration of the dye before and after the experiment was found to remain unchanged and approximately the same results were obtained.

The following samples of methylene blue were employed, which according to the writer's knowledge represent some of the purest available.

(1) $C_{16}H_{18}N_35Cl + 1H_2O$ sent by Dr. Benda from Germany.

(2) $C_{16}H_{18}N_35Cl + 3H_2O$ sent by Dr. Benda from Germany.

(3) Sample F and Sample G, given by Dr. W. M. Clark and Dr. B. Cohen of the Hygienic Laboratory, Washington, D. C

(4) Bleu de methylen pour Bacteriologie, Microbiologie, Physiologie, Produit, given by Dr R. H. French of the Color Laboratory, United States Department of Agriculture, Washington, D. C.

(5) Merck's medicinal.

Owing to the difference in the solubility of different samples in sea water, different concentrations[12] varying from 0 01 to 0 04 per cent were employed.

In determining the concentration of dye in the sap the following method was used. Sap was collected by puncturing the cell (previously removed from the dye solution and wiped) by means of a sharp glass capillary tube, and by drawing up the sap from the vacuole. About 2 cc. of sap was then placed in a small test-tube and the color of this test-tube was matched with that of a test-tube of the same diameter containing a known concentration of methylene blue.

Merck's medicinal was used as the standard solution for all the samples employed because this was the only sample available in sufficient quantity to make up a series of standard solutions at different concentrations. When the concentration of dye in the sap was below 0.00004 per cent, the color appeared more greenish than the standard so that it was difficult to match the color. Furthermore, above 0 0003 per cent the color of the test-tube containing the sap appeared more purplish than that of the standard so that it again became difficult to match.

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[12] Other concentrations were used as check experiments. In all cases, if any dye entered the vacuole of uninjured cells more entered from the external solution at pH 9.5 than at 5 5, provided the experimental errors described in the text are absent.

Experiments were carried out at 25° ± 0.5°C. in an incubator with air holes through which diffused light was allowed to enter.

When living cells of *Valonia* were placed in methylene blue dissolved in sea water at these two pH values it was found that at about pH 5.5 practically no dye penetrated the vacuole, while at about pH 9.5 more entered. For example, with Merck's medicinal methylene blue, after 1 hour, at pH 9.5, the concentration of dye in the sap was about 0.00006 per cent, while at pH 5.5 it was too dilute for determination. When other samples of methylene blue, already described, were used, it was found that with some samples more dye entered than from the Merck's medicinal, while from others less entered the vacuole. But in all cases the rate of penetration of the dye into the vacuole was higher with the external dye solution at pH 9.5 than at pH 5.5. But with the samples in which the penetration was extremely slow the amount of dye found in the vacuole was so small even after several hours of exposure that unless extreme care was taken there were possibilities of experimental error arising from (1) contamination of the sap from dye in the cell wall at the time of puncturing exceeding the actual penetration of dye into the vacuole before puncturing; (2) inability to match the color of the test-tubes accurately; (3) more rapid penetration of dye due to a slight and reversible injury which cannot be detected, and which may occur if experiments are extended for several hours or if the cells at the start are not in excellent condition (the dye enters more rapidly as the cells become injured).

These sources of error might in some cases cause the rate of penetration of dye to appear the same at pH 5.5 and at pH 9.5. Furthermore, since at pH 5.5 the cells become injured more rapidly than at pH 9.5, in some cases where the injury occurred to the extent of a very slight loss of turgidity the rate of penetration at pH 5.5 was found to be higher than that at pH 9.5.

Experiments have been made with cells which have been kept in stoppered glass bottles containing some sea water for several months, as well as with cells which have been kept in a pan of sea water for several weeks. In both cases it was found that so long as the cells were not injured, the dye entered more rapidly at pH 9.5 than at pH 5.5, though in the case of the cells which have been kept in the laboratory for several months, as described above (cells appeared less green than more recently collected cells), the dye entered more rapidly than in the case of the cells which have been kept only for a few weeks.

When the sap collected from the uninjured cells which had been exposed to the dye solution was oxidized by shaking and exposing to air with an alkaline reaction, no increase in coloration took place so that we may conclude that there was no dye in reduced form present in the sap.

When the pH value of the sap was determined after the living cells of *Valonia* had been exposed to sea water at pH 5.5, 9.5, and 10.9 for 4 hours, no change in the pH value of the sap occurred if the cells were not injured.

III.

Spectrophotometric Analysis.

The nature of the dye in the external solution and in the vacuolar sap of uninjured and injured cells was tested by means of spectrophotometric determinations. The measurements were made at the Color Laboratory in Washington, D. C., by W. C. Holmes, of whose collaboration the writer desires to express her appreciation.

The instrument employed was a Hilger wave-length spectrometer, equipped with a Nutting photometer. Either 1 or 2 cm. layers of solution were examined, depending on the concentration of dye in the solutions and the quantities of solution available. The measurements were carried out over the spectral range between 540 and 690 mμ. The concentration of dye was adjusted, insofar as was possible, to afford maximum visual sensitivity at and near the absorption maximum of the dye in dilute aqueous solution. All recorded values in this restricted region are averages of a considerable number of measurements.

A brief statement of spectroscopic criteria is advisable at this point. The absorption maximum of methylene blue in dilute aqueous solution is approximately 665 mμ. The corresponding maximum of trimethyl thionine is approximately 650 mμ. Although the average visual sensitivity in this region of the spectrum is relatively inferior it is readily possible to locate absorption maxima (with favorable dye concentrations) within a possible variation of about ±1mμ.

The determination of the approximate absorption maximum, accordingly, differentiates the two dyes with absolute certainty. It affords, moreover, a reliable, if somewhat rough, criterion of the relative proportions of the dyes in question when both are present. Owing to the relatively limited spectral interval between their bands the band of a mixture of the dyes does not inhibit the individual maxima of its two component bands, but, rather, a single composite maximum of which the location varies with dye proportions. The absorption maximum of a mixture containing 66 per cent of methylene blue and 33 per cent of trimethyl thionine, for example, falls at approximately 660 mμ, while that of a mixture of 33 per cent of methylene blue and 66 per cent of trimethyl thionine falls at approximately 655 mμ.

It may be noted that the employment of suitable spectrophotometric ratios would afford a more exact definition of relative dye proportions. The basic data requisite for this procedure, however, were not available when the present investigation was begun and it was felt that the mere determination of the approximate absorption maxima of solutions would afford ample evidence of their character for present purposes.

Both methylene blue and trimethyl thionine exhibit secondary absorption in the general spectral region near 600 mμ. Both dyes are held to exist in aqueous solutions in a state of tautomerism between two dye forms.[13] In the present investigation considerable variations were noted in the apparent tautomeric equilibria between dye forms. These arise primarily from variations in dye concentration and are also influenced by other factors. A discussion of these phenomena is unnecessary in this paper. It is sufficient to note that their occurrence does not modify in any appreciable degree the relative absorption of the dyes at different wave-lengths within the critical spectral region between 650 and 665 mμ, or invalidate conclusions derived from variations in absorption within that region.

When such analyses were made some very interesting facts were obtained, as follows:

I. A sample of methylene blue,[14] dissolved in (1) Bermuda sea water, (2) sap of *Valonia macrophysa*, and (3) artificial sap,[15] gave absorption maxima[16] characteristic of methylene blue, about 665 mμ (see Table I and Fig. 1, Curves *A*, *B*, and *C*).

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[13] Holmes, W. C., *Ind. and Eng. Chem*, 1924, xvi, 35, *Stain Technol*, 1926, i, 17.

[14] Several samples were employed (see Section II in text).

II. The dye allowed to diffuse out of the cell wall (which had been previously stained by placing the living cells for a few minutes in sea water containing methylene blue), into artificial sap of *Valonia* gave the absorption maximum of 665 mμ characteristic of methylene blue (Table I and Fig. 1, Curve *D*).

TABLE I.

Solutions	Primary absorption maximum
	mμ
Methylene blue dissolved in sea water	665
Methylene blue dissolved in the sap of *Valonia*	665
Methylene blue dissolved in artificial sap of *Valonia*	665
Dye which has diffused out of the cell wall into artificial sap, after the cell wall of living cells of *Valonia* has been stained in methylene blue (dissolved in sea water)	665
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5	663
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 5.5	665
Trimethyl thionine dissolved in sap of *Valonia*	650
Dye found in the vacuole of uninjured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 9.5: This was freed from chloroform by absorbing it in distilled water	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 5.5: This was freed from chloroform by absorbing it in distilled water	655
Methylene blue dissolved in distilled water	665
Dye absorbed by chloroform from methylene blue dissolved in M/150 buffer mixtures at pH 5.5 or at pH 9.5: This was freed from chloroform by absorbing it in distilled water	650

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[15] The pH value of the sap is about 5.8. The sap contains about 0.6 M halides (*cf.* Osterhout, W. J. V., and Dorcas, M. J., Foot-note 11).

[16] The absorption curve thus obtained resembles that of a higher concentration of methylene blue dissolved in distilled water. This may be due to the effect of salt on the dye, as suggested by Dr. W. C. Holmes.

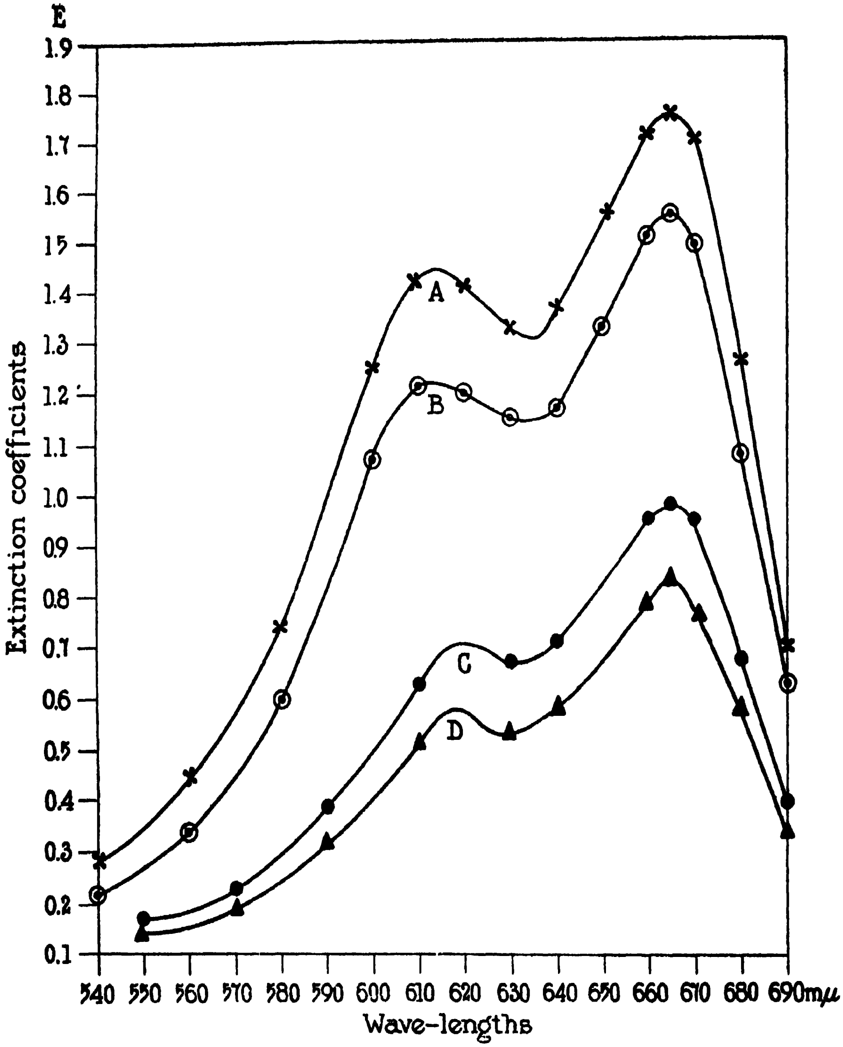

FIG. 1. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curve *A* represents the methylene blue dissolved in sea water, Curve *B* in the sap of *Valonia*, Curve *C* in the artificial sap of *Valonia*, Curve *D* the dye that has diffused from the cell wall into artificial sap after the cell wall of living cells of *Valonia* has been stained in methylene blue dissolved in sea water.

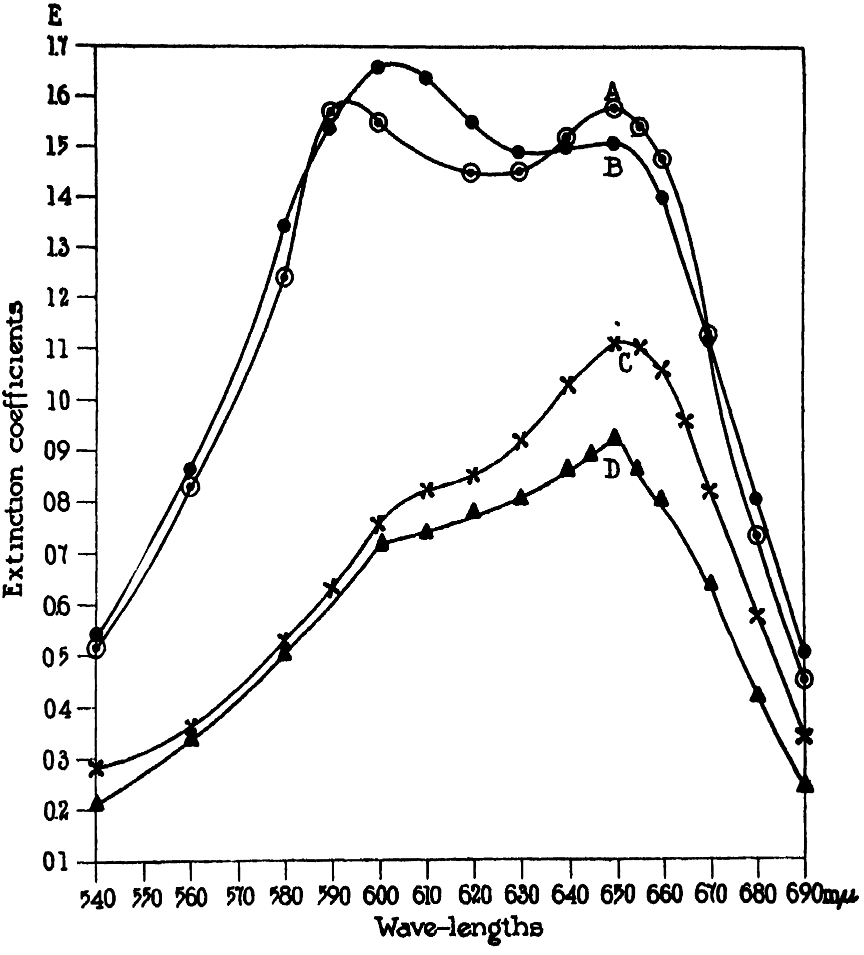

FIG 2 Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves *A* and *C* (symbols ⊙ and ×) represent the dye which was found in the vacuolar sap of uninjured cells of *Valonia* after the cells have been stained for 1 hour in one sample of methylene blue dissolved in sea water at pH 9 5 (Curve *A*) and in another purer sample at pH 10 9 (Curve *C*). Curves *B* and *D* represent the sample of trimethyl thionine or azure B dissolved in sap of *Valonia* at two dilutions, corresponding approximately to two dilutions represented by Curves *A* and *C* respectively. Curve *B* corresponds with Curve *A* and Curve *C* with Curve *D*.

III. Two concentrations of trimethyl thionine or azure B (sent by W. C. Holmes) dissolved in the sap of *Valonia* (Table I, Fig. 2, Curves *B* and *D*) gave absorption maxima of 650 mμ. This sample is obtained by the oxidation of methylene blue. Holmes suggests that though it is shown to be a fairly pure product it is possible that it contains small proportions of both methylene blue and asymmetrical dimethyl thionine.

IV. The dye in the sap collected from the vacuole of uninjured cells after an exposure of 1 hour to sea water saturated with two samples of methylene blue[17] (1) 0.04 per cent at pH 9.5 (Curve *A*, Fig. 2), and (2) 0.01 per cent at pH 10.9 (Curve *C*, Fig. 2). In Curve *A* the absorption maximum is 650 mμ which shows that the dye is chiefly trimethyl thionine and it gives no visible evidence of the presence of methylene blue. The absorption maximum of Curve *C* is about 652 mμ which shows that there is a trace of methylene blue, though the dye is chiefly trimethyl thionine. The presence of a trace of methylene blue in all probability is due to the contamination of the sap from the stained cell wall at the time the cell was punctured to collect the sap. Such a contamination plays an important part whenever the concentration of the dye in the sap is relatively small.

V. The dye collected from the vacuole of injured cells (slightly soft) after 12 hours' exposure to sea water saturated with methylene blue, either at (1) pH 5.5, or (2) at pH 9.5 gave an absorption maximum of 665 mμ (methylene blue) for (1) and 663 mμ (methylene blue and a little azure B) for (2) (Table I, and Fig. 3, Curves *A* and *B*). Since in both cases the dye in the sap collected from the vacuole was diluted with the sap collected from the vacuole of unexposed living cells, the heights of the curves which vary with dilution (the higher curve corresponding to the higher concentration) given in Fig. 3 do not show true relative concentrations of the dye found in the vacuole.

The azure B found by spectrophotometric analysis in the sap collected from the vacuole of uninjured cells of *Valonia* is not due to the transformation of methylene blue into azure B after methylene blue has penetrated from the external dye solution into the vacuole because not enough conversion takes place during 1 to 3 hours in the methylene blue dissolved in the sap of *Valonia* to be detected by this method.

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[17] Samples employed are specified in the text in Section II. Owing to the fact that the purpose of these experiments is not to determine the purity of these samples, the sample used for each result is not specified.

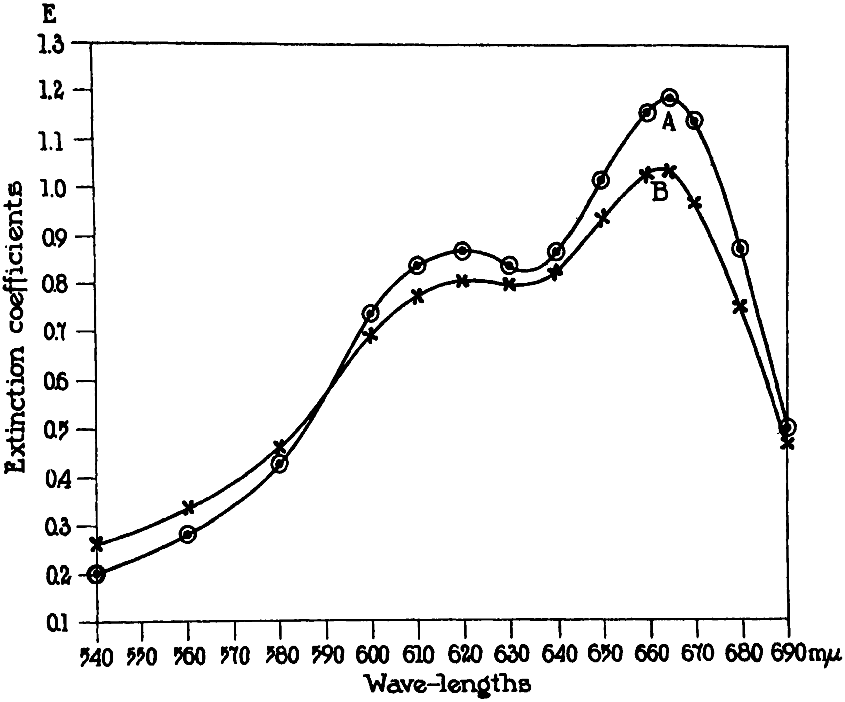

FIG. 3. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves representing the dye in the sap collected from the vacuole of injured cells of *Valonia* and diluted with sap after the cells have been stained in the methylene blue dissolved in sea water, Curve *A* at pH 5.5 and Curve *B* at pH 9.5.

IV.

Absorption of Dye by Chloroform from Methylene Blue Solution.

In view of the fact that a similarity was found between *Valonia* and chloroform in their behavior toward other basic[18] dyes, in that

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[18] When living cells of *Valonia* were placed in different basic dyes, Lauth's violet, neutral red, and brilliant cresyl blue, it was found that the higher the pH values of the dye (*viz.* between pH 5 and pH 8), the more rapidly the dye

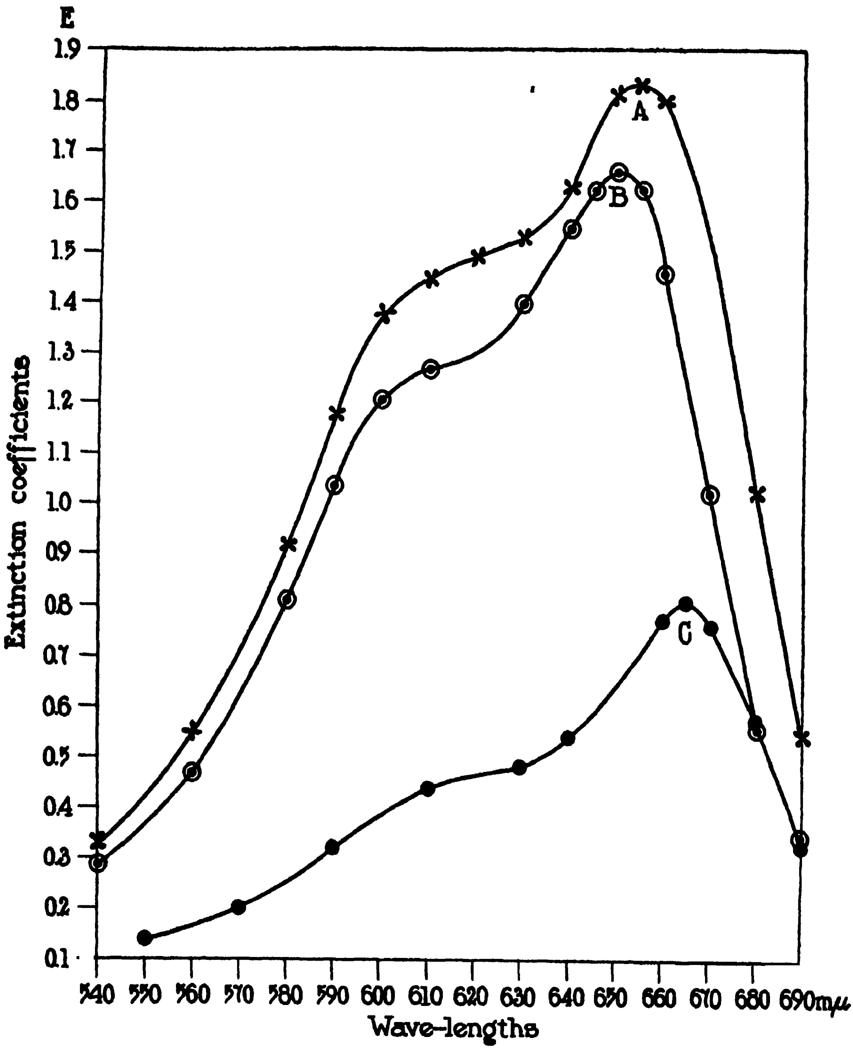

FIG. 4. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves *A* and *B* represent the dye which was absorbed by chloroform from methylene blue dissolved in sea water, Curve *A* at pH 5.5 and Curve *B* at pH 9. In both cases the dye was freed from chloroform by its subsequent absorption in distilled water. Curve *C* represents methylene blue dissolved in distilled water.

both take up the dye in the form of free base much more readily than in the form of salt, it seemed possible that chloroform, like *Valonia*, takes up chiefly trimethyl thionine or azure B from a solution of methylene blue, which may be determined by means of spectrophotometric analysis and by the determination of the partition coefficient of the dye between chloroform and water.

Heretofore no spectrophotometric analysis of the dye absorbed by chloroform from methylene blue has been made. This was accordingly done in the case of the dye absorbed by chloroform from a solution of methylene blue (made up in sea water). From chloroform thus stained, dye was freed by subsequent absorption in distilled water. When the sea water was at pH 9.5, the dye absorbed by chloroform gave the absorption maximum 650 mμ, characteristic of azure B (Table I, and Fig. 4, Curve *B*), and at pH 5.5 an absorption maximum of 655 mμ (which showed that there was a small amount of methylene blue in addition to azure B, Table I, and Fig. 4, Curve *A*). The methylene blue dissolved in distilled water gave an absorption maximum of 665 mμ (Table I, Fig. 4, Curve *C*).

The dye absorbed by chloroform from aqueous methylene blue solution (made up with M/150 buffer mixtures) was found to be azure B, both at pH 5.5 and at pH 9.5 (Table I).

These analyses show that in the presence of sea water at pH 5.5 azure B and a small amount of methylene blue are absorbed by chloro-

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entered the vacuole. Relative rate of penetration differed with various basic dyes. Such differences in the rates corresponded roughly with the differences in the degree of absorption of these dyes by chloroform at different pH values of the sea water, and in the basicity of the dyes. When the amount of brilliant cresyl blue absorbed by chloroform or by *Valonia* is plotted against the external pH values, an S-shaped curve is obtained in both cases.

In view of the fact that in presence of sea water a basic dye in form of salt, as well as in form of free base, enters the chloroform, it is difficult to obtain with any accuracy the distribution coefficient of the dye only in form of free base between chloroform and sea water. This complication, however, was absent in the case of the basic dyes dissolved in M/150 buffer solutions, so that it was possible to make a comparison on a quantitative basis between the absorption of dye in form of free base by the vacuole of *Nitella* and by chloroform (Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 561).

form, while from sea water at pH 9.5 and from dilute buffer solutions at both pH values chiefly azure B is absorbed.

As to the experiments on the distribution of the dye between methylene blue dissolved in sea water and chloroform the following results were obtained.

When 3 cc. of chloroform were shaken up with 10 cc. of 0.002 per cent methylene blue dissolved in sea water at pH 9.5 and pH 5.5, it was found that the apparent partition coefficient of the dye between chloroform and sea water at pH 9.5 was about 2 (*i.e.* more concentrated in chloroform), while at pH 5.5 it was about 1.3. These might be called the apparent partition coefficients, since they merely represent the ratio of the concentration of the dye in the chloroform to that of the dye in sea water, without taking into account whether the dye is azure B, methylene blue, or a mixture of both.

The color of the dye in the chloroform when absorbed from methylene blue in sea water at pH 9.5 is reddish purple and from methylene blue in sea water at pH 5.5 is blue. Since the dry salt of methylene blue and that of azure B dissolved in chloroform is blue, the dye in the chloroform absorbed from methylene blue in sea water at pH 5.5 may possibly represent a mixture of methylene blue and azure B, in form of salt, while the dye in chloroform absorbed from methylene blue in sea water at pH 9.5 is azure B chiefly in form of free base. These experiments are insufficient to show whether or not these dyes in form of salt enter chloroform as undissociated molecules which may possibly be formed to a certain extent in presence of so high a concentration of salt such as sodium chloride in the sea water.

Just as in the case of the vacuole of uninjured cells of *Valonia* the dye which is taken up by chloroform from methylene blue in sea water at pH 9.5 is chiefly azure B in form of free base and not methylene blue.

Whether methylene blue is capable of penetrating the vacuole from methylene blue in sea water at pH 5.5 cannot be determined, since the dye does not penetrate in sufficient quantity for spectrophotometric analysis. Since the concentration of undissociated molecules of methylene blue in form of salt possibly present in sea water is not determined, this result neither proves nor disproves the theory that the undissociated molecules enter the vacuole of uninjured cells more rapidly than the ions.

It is not certain as to whether methylene blue enters the chloroform in constant amount at all pH values or only at lower pH values. Further experiments are necessary to determine this point.

V.

Penetration of Azure B into the Vacuole of Valonia and into Chloroform.

If our supposition is correct that azure B is a weaker base than methylene blue, and capable of existing in form of free base at higher pH values, then, according to the theory presented in Section I, a pure sample of azure B should penetrate into the vacuole of *Valonia* and into chloroform more when the pH value of the sea water is higher.

Living cells of *Valonia* were therefore placed for ½ hour in 0.04 per cent azure B (1) made by Holmes, (2) extracted by chloroform from methylene blue solution (made up with borate buffer at pH 9.5). With both samples approximately the same results were obtained, in that at pH 9.5 the rate of penetration was much higher (about 0.001 per cent dye in sap) than at pH 5.5 (dye in sap was too dilute for accurate determination).

When 3 cc. of chloroform were shaken up with 10 cc. of the azure B dissolved in sea water at pH 9.5 and at pH 5.5, the partition coefficient of the azure B (made by Holmes) between chloroform and sea water at pH 9.5 was 14.9 and at pH 5.5 was 1.8 (*i.e.* the dye was more soluble in chloroform than in sea water).

The color of the dye in chloroform when sea water was at pH 9.5 was reddish purple, while it was blue at pH 5.5.

Since the dry salt of azure B dissolved in chloroform appears blue, the azure B taken up by chloroform from sea water at pH 5.5 may be in form of salt, and at pH 9.5 in form of free base (which is reddish purple).

VI.

DISCUSSION.

From these results we may conclude that the vacuole of uninjured cells of *Valonia macrophysa* takes up chiefly trimethyl thionine (azure B) from the solution of methylene blue which contains so little azure B (as impurity) that it cannot be detected by the spectrophotometer. As soon as cells are injured methylene blue enters. The writer's results and conclusion are contrary to those obtained by M. M. Brooks,[19] who states that the vacuole of uninjured cells of *Valonia macrophysa* takes up dye from methylene blue solution with the same speed at all pH values (from pH 5 to 9), from which she concludes (without analysis of the dye in the vacuole) that methylene blue (in form of salt) enters the vacuole of uninjured cells.

The writer's experiments show that the penetration of dye into the vacuole of uninjured cells from a solution of methylene blue does not discredit the theory that the basic dye enters the vacuole chiefly in the form of free base, since the dye which penetrates is found to be chiefly a lower homologue of methylene blue, azure B, which is less basic and capable of existing in part in the form of free base at higher pH values. Azure B behaves like all other basic dyes in that its relative rate of penetration depends on the amount of dye in form of free base present, which corresponds with the pH value of the external solution (the higher the pH value the more dye is in form of free base and the more rapid is the rate of penetration). That this difference in the rate of penetration at varying pH values is not due primarily to the effect of different pH values on the protoplasm is shown by the fact that the relative rates of penetration at a given series of pH values differ with different basic dyes.

The writer's previous statement that the vacuole of living cells, such as that of *Valonia*, behaves very much like chloroform toward basic dyes, in that they both take up the dye in the form of free base, is still further supported by the fact that they both take up primarily azure B from methylene blue solution at higher pH value.

Undoubtedly the penetration of a basic dye depends chiefly on two factors under such experimental conditions, (1) on the apparent dissociation constant of the dye, (2) on the partition coefficient of the dye between the vacuolar sap and the external solution, and in some cases on that of the dye between the vacuolar sap and the protoplasm. In case there is a combination of dye with some constituent of the sap, this factor must be brought into consideration. With chloroform also penetration depends on the dissociation constant and the partition coefficient.

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[19] Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

There is a similarity between chloroform and the vacuole of uninjured cells of *Valonia* in that they are both capable of taking up azure B and some other basic dyes in form of free base, but in certain cases chloroform and *Valonia* are found not to behave alike. For example, some acid dyes are slightly soluble in chloroform but they do not penetrate the vacuole of uninjured cells of *Valonia.* However such an analogy is not complete since the ability of the dye to collect in the vacuole may not only depend on the ability of the protoplasmic layer (between protoplasm and external solution, or between protoplasm and the vacuole) to absorb the dye but also on its power to give up the dye. Experiments are being done with this consideration in view.

The fact that azure B instead of methylene blue is found in the vacuole is not proof that methylene blue does not enter the protoplasm. It might enter the protoplasm though it does not penetrate into the vacuole. One way to arrive at a definite conclusion is to determine the nature of the dye inside the protoplasm, after the dye has been allowed to penetrate the cell in uninjured condition; but with the protoplasmic layer of *Valonia* this cannot be accomplished since it cannot be removed for examination without contamination or injury. Furthermore there is no way of determining whether or not methylene blue enters protoplasm and is converted to azure B or trimethyl thionine.

These experiments show the danger of drawing conclusions as to permeability or as to oxidation-reduction potentials from the experiments on the penetration of dye from a solution of methylene blue into living cells, unless we know the nature of the dye both in the external solution and inside the cells.

These experiments were repeated with *Nitella* and gave approximately the same results.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.

SUMMARY.

When uninjured cells of *Valonia* are placed in methylene blue dissolved in sea water it is found, after 1 to 3 hours, that at pH 5.5

practically no dye penetrates, while at pH 9.5 more enters the vacuole. As the cells become injured more dye enters at pH 5.5, as well as at pH 9.5.

No dye in reduced form is found in the sap of uninjured cells exposed from 1 to 3 hours to methylene blue in sea water at both pH values.

When uninjured cells are placed in azure B solution, the rate of penetration of dye into the vacuole is found to increase with the rise in the pH value of the external dye solution.

The partition coefficient of the dye between chloroform and sea water is higher at pH 9.5 than at pH 5.5 with both methylene blue and azure B. The color of the dye in chloroform absorbed from methylene blue or from azure B in sea water at pH 5.5 is blue, while it is reddish purple when absorbed from methylene blue and azure B at pH 9.5. Dry salt of methylene blue and azure B dissolved in chloroform appears blue.

It is shown that chiefly azure B in form of free base is absorbed by chloroform from methylene blue or azure B dissolved in sea water at pH 9.5, but possibly a mixture of methylene blue and azure B in form of salt is absorbed from methylene blue at pH 5.5, and azure B in form of salt is absorbed from azure B in sea water at pH 5.5.

Spectrophotometric analysis of the dye shows the following facts.

1. The dye which is absorbed by the cell wall from methylene blue solution is found to be chiefly methylene blue.

2. The dye which has penetrated from methylene blue solution into the vacuole of uninjured cells is found to be azure B or trimethyl thionine, a small amount of which may be present in a solution of methylene blue especially at a high pH value.

3. The dye which has penetrated from methylene blue solution into the vacuole of injured cells is either methylene blue or a mixture of methylene blue and azure B.

4. The dye which is absorbed by chloroform from methylene blue dissolved in sea water is also found to be azure B, when the pH value of the sea water is at 9.5, but it consists of azure B and to a less extent of methylene blue when the pH value is at 5.5.

5. Methylene blue employed for these experiments, when dissolved

in sea water, in sap of *Valonia*, or in artificial sap, gives absorption maxima characteristic of methylene blue.

Azure B found in the sap collected from the vacuole cannot be due to the transformation of methylene blue into this dye after methylene blue has penetrated into the vacuole from the external solution because no such transformation detectable by this method is found to take place within 3 hours after dissolving methylene blue in the sap of *Valonia*.

These experiments indicate that the penetration of dye into the vacuole from methylene blue solution represents a diffusion of azure B in the form of free base. This result agrees with the theory that a basic dye penetrates the vacuole of living cells chiefly in the form of free base and only very slightly in the form of salt. But as soon as the cells are injured the methylene blue (in form of salt) enters the vacuole.

It is suggested that these experiments do not show that methylene blue does not enter the protoplasm, but they point out the danger of basing any theoretical conclusion as to permeability on oxidation-reduction potential of living cells from experiments made or the penetration of dye from methylene blue solution into the vacuole, without determining the nature of the dye inside and outside the cell.

UDDER SIZE IN RELATION TO MILK SECRETION.

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(Accepted for publication, May 31, 1927.)

Our knowledge of the mammary gland as related to its size and function has been largely based on the observational relationship which exists between udder size and the milk the cow is able to give.[1] Briefly considered the data available consist of two measurements, the milk production of the cow in pounds and the relative degree of perfection of the udder in size, shape, and quality as measured on an arbitrary scale called her score. Study of this information on 1674 Jersey cattle shows a correlation between the size and quality of the udder and the milk yield which the cow was able to produce. This correlation, while markedly significant, is low, .19 ± .016. The evidence thus points to the conclusion that despite the obvious sources of error the size of the udder is a function at least of the milk which the cow is able to secrete. The just published work of Gaines and Sanmann[2] supports this hypothesis while the work of Maxwell and Rothera[3] and the opinions of many dairymen and veterinarians citing the size of the udder as being too small to hold the milk the cow is able to give at any one time controvert it.[4] Stated quantitatively the problems before us are: (*a*) what proportion of the milk given by the cow at milking is already stored in the udder; (*b*) what correlation exists between udder size and milk yield; (*c*) how much secreting tissue is necessary to manufacture a pound of milk; (*d*) what is the relation between secreting and supporting tissue in the udder.

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[1] Gowen, J. W., Conformation and its relation to milk-producing capacity in Jersey cattle, *J. Dairy Sc.*, 1920, iii, 1. Studies on conformation in relation to milk-producing capacity in cattle. II. The personal equation of the cattle judge, *J. Dairy Sc.*, 1921, iv, 359. Studies on conformation in relation to milk-producing capacity in cattle. III. Conformation and milk yield in the light of the personal equation of the dairy cattle judge, *Annual Rep. Maine Agric. Exp. Station*, 1923, 69.

[2] Gaines, W. L., and Sanmann, F. P., The quantity of milk present in the udder of the cow at milking time, *Am. J. Physiol.*, 1927, lxxx, 691.

[3] Maxwell, A. L. I., and Rothera, A. C. H., The action of pituitrin on the secretion of milk, *J. Physiol.*, 1914–15 xlix, 483.

For the work herein cited nine dairy cows were used. These cows were milked twice a day, the time of the first milking being 1.00 p.m., and that of the second milking, 10.00 p.m. The cows were milked on these hours for 5 days before they were killed. Their milk was weighed after each milking and a sample of the milk taken for the analysis of the lactose content. The cows were then shipped 10 miles to a slaughter house where they were killed at 1.00 p.m.[5] The technique was varied for the first two cows. The udder of one of these cows was minced and the minced material subjected to a pressure of 2000 pounds in an hydraulic press. This technique proved unfortunate in view of the fact that the udder material holds the milk secreted very tightly, it being almost impossible to press out any of the milk although it may be milked or drained out. In consequence the minced material was simply driven into the cloth and had to be extracted with water in the same manner as that used for the later seven cows. The second cow's udder was cut into small strips and allowed to drain and then these strips were put into the ton press. Here again it was impossible to press out any amount of secretion. The material was then extracted with water in the manner described for the other seven cows. The results of this unfortunate technique showed clearly that the musculature of the udder is such that milk may be extracted from it far more easily by the ordinary methods of milking with the cooperation of the cow than is possible with relatively large pressures applied under external conditions. The technique for the other seven cows consisted of milking these cows at 1.00 p.m. and 10.00 p.m. for 5 consecutive days, determining the milk flow and lactose percentage in the milk for each of these milkings. The cows were then killed at exactly the hour of previous milking, the last three being killed after milking, the other four being killed with the udder full of milk. The udders were then carefully dissected off, cut in strips, and drained for the milk which would quickly flow out. The remaining material was then ground and extracted with water three different times, about

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[4] That this opinion lacks foundation in fact in at least five cows is shown by the work of Swett, W. W., Relation of conformation and anatomy of the dairy cow to her milk and butterfat-producing capacity. Udder capacity and milk secretion, *J. Dairy Sc.*, 1927, x, 1.

[5] It is a pleasure to acknowledge our indebtedness to Penley's Packing Company, Auburn, Maine, for their cordial cooperation.

50 pounds of water being used in each extraction, the fluid material being drained through cheese-cloth bags. The remaining extracted udder material was then dried, ground, and the little sugar which remained in it was determined by first removing the fat with gasoline and then extracting with water. Printing cost allows only publication of the totals.

Table I shows the milk production of the cows for the 1 o'clock milking and the 10 o'clock milking for the 3 days previous to their slaughtering.

TABLE I.

Average Milk Production in Pounds for the 1 p.m. and 10 p.m. Milking, for the 3 Days Previous to Slaughter. August, 1926.

Cow No.	Average milk yield	
	1 p. m.	10 p. m.
111	12.9	7.4
124	17.9	10.0
132	15.6	10.3
136	15.7	13.8
148	13.5	8.3
154	11.7	5.9

TABLE II.

Average Milk Production in Pounds for the 1 p.m. and 10 p.m. Milking of Cows Slaughtered Just Following 1 p.m. Milking. August, 1926.

Cow No.	Average milk yield		
	1 p. m.	10 p. m.	Last 1 p. m. milking
97	4.7	2.2	2.8
114	4.1	2.8	3.6
153	10.8	6.9	9.6

Table II shows the milk production of the cows, at the 1 o'clock and 10 o'clock periods, which were slaughtered just following milking. The milk productions in Table II are lower than those in Table I. The cows had to be shipped 10 miles to the slaughter house before they were killed, so that this disturbance probably played some part in their milk production before slaughter, tending to decreased secretion and to retention of that which was secreted. This is noted in the fact that the milk production of Cow 97 was 1.9 pounds less than her 1 o'clock average for the 3 previous days. No. 114 had 0.5 of a pound less than the average of the 3 previous days, and No. 153, 1.2 pounds less than the average of her earlier milkings. It was found that 0.8 of a pound of milk could be drained out of the udder of No. 97, although the milking had been performed very carefully. Cows 114 and 153 showed some milk left in the udder, but not enough to weigh. This milk was allowed to go into the determination of the sugar content in the udder, following milking.

TABLE III.

Average Lactose Per Cent for 1 p.m. and 10 p.m. Milking for 3 Days Previous to Slaughter. August, 1926.

Cow No.	Average lactose	
	1 p. m.	10 p. m.
	per cent	*per cent*
111	4.62	4.60
124	4.78	4.72
132	5.28	5.33
136	5.05	5.10
148	4.81	4.89
154	4.84	4.90

TABLE IV.

Average Lactose Per Cent for 1 p.m. and 10 p.m. Milkings of Cows Slaughtered Just Following 1 p.m. Milking. August, 1926.

Cow No.	Average lactose		Lactose
	1 p. m.	10 p. m.	Last 1 p. m.
	per cent	*per cent*	*per cent*
97	3.98	4.03	3.80
114	4 72	4.80	4.85
153	4.50	4.58	4.50

Tables III and IV show the percentages of lactose found in the daily milkings of the cows given in Tables I and II. It will be noticed that there appears to have been little or no change in the lactose percentages of Cows 97, 114, and 153 from the average for the milk of the 3 previous days. It may be concluded from this that any excitement incident to the trip to the slaughter house for these cows had no effect in changing the lactose content of their milk.

The total lactose for all of the extractions is shown in Table V. This total lactose divided by the average lactose percentage for the 1 o'clock milk yield of the 3 preceding days gives the amount of milk necessary to account for this total amount of lactose. Throughout, the materials added checked well with those extracted. The udder material remaining after extraction shows an average of less than .04 per cent lactose.

TABLE V.

Weight of Lactose Extracted from Udders and Milk Equivalent of Lactose, Pounds. August, 1926.

Cow No.	Weight of total lactose	Milk equivalent
111	.718	15.5
124	.931	19.5
132	.774	14.6
136	.798	15.6
148	.614	12.8
154	.538	11.2
97	.247	6.2
114	.140	3.0
153	.150	3.3

The lactose extracted from the udder, in the case of the unmilked cows, represents the lactose contained in the milk which would be drawn on milking and the lactose which would remain in the udder. For the cows milked just before death the lactose represents the milk which could not be drawn from the udder by milking. This, converted into pounds of milk, represents for Cow 97, 6.2 pounds; for Cow 114, 3.0 pounds; for Cow 153, 3.3 pounds. The large amount of milk remaining in the udder of Cow 97 calls for comment. After the milking was completed and the udder dissected off about 3/4 of a pound of milk was found in this udder which could easily be drained out of it. This milk was held up by the cow even though the milking was performed carefully by an experienced milker. It will be noticed that no such amount of milk was found in the udders of the other two cows, 114 and 153. In fact, not over .1 of a pound of easily drained milk was left in these udders. Should the 3/4 of a pound of free milk be left out of consideration there is still 5.4 pounds found in this udder. The total milk of Cow 97 is consequently much larger than her previous milk yield would lead one to expect. How this cow manufactured this extra 2 or 3 pounds of milk the authors do not know. These three cows' milk yields before killing, in comparison with the average of the 3 previous days, were 1.9 pounds less than would be expected for Cow 97, .5 of a pound less for Cow 114, and 1.2 pounds less for Cow 153. The reduction in milk yield seems to be accounted for by the strange conditions under which the milking took place and the nervous excitement previous to milking. If these values be subtracted from the milk found in the udder, determined as lactose, Cow 97 has 4.3 pounds of milk remaining in the udder; Cow 114, 2.5 pounds; and Cow 153, 2.1 pounds. Cows 114 and 153 appear to check nicely. Cow 97 has about 2 pounds more milk in the udder than would be expected on the basis of the other results. Whether the average of the three cows should be used or only the average of the last two is perhaps a question. If the average of the three cows is used it is found that the udder contains 3 pounds of milk when it is supposedly milked dry. The milk found in the udders of the six remaining cows determined as the lactose equivalent, represents the milk which would be drawn in normal milking plus that which was retained in the udder after the cow was considered dry. If these figures be compared with the amount of milk which the cows gave it will be noted that they correspond fairly well. Thus the average milk production of Cow 111 was 12.9 pounds while the amount accounted for is 15.5 pounds, that for Cow 124 was 17.9 pounds while the milk accounted for is 19.5 pounds, that for Cow 132 was 15.6 pounds while the milk accounted for is 14.6 pounds, for Cow 136 the milk production was 15.7 pounds while the milk accounted for is 15.6 pounds, for Cow 148 the milk production was 13.5 pounds while that accounted for was 12.8 pounds, and finally the milk production of Cow 154 was 11.7 pounds while that accounted for was 11.2 pounds. It will be noted throughout that the amount of milk accounted for and the amount of milk drawn from the udder correspond fairly closely. They show, furthermore, the following relative relationships: the higher milking cows show the larger amount of milk in their udders; the lower milking cows, the lower amount of milk in their udders; with the medium yielding cows between the two extremes. The average milk production for the six cows was 14.6 pounds; the average amount of milk accounted for for these cows was 14.9 pounds. It is to be remembered that of this milk accounted for on the basis of lactose there is probably remaining in the udder after milking between 2 and 3 pounds of milk. Thus from the 14.9 pounds accounted for on the basis of lactose there should be subtracted between 2 and 3 pounds due to the amount of milk which it is impossible to milk from the udder. The comparison of the amount of milk accounted for should be therefore between 12 and 13 pounds as contrasted with the 14.6 obtained. All these results show clearly that at actual time of milking between 80 and 85 per cent of the milk can be accounted for in the udder of cows milking up to 30 pounds of milk a day.[6] The experiment therefore points to the conclusion that 20 per cent is a maximum and 10 to 15 per cent, a probable value for the amount of milk which may possibly be secreted in the udder during the time of milking.

This value is considerably less than that obtained by Maxwell and Rothera in their experimental work. Their results are largely dependent upon the accuracy with which the lactose in the milk of the cat is represented by their assumed value of 5.07 per cent. Gaines and Sanmann cite Folin, Denis, and Minot's analyses on 19 samples of the milk of three cats. These data give a range in percentage of lactose from 2.3 to 4.0. For 26 samples on four cats the average was 3.4. Should 3.4 be used in place of 5.07 as the lactose per cent of cats' milk, the total secretion of the mother cat as extracted by the kittens is accounted for.

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[6] The technique of the experiment is such that it is necessary to make the sugar analysis over a period up to 4 days from the time of extracting the udder. The extracted materials were carefully preserved on ice and 10 drops of formalin added to each pint of extract. This method of preservation has shown little change in the sugar content although that possibility is to be considered. The acidity test was throughout all of the experimental work between .005 and .19 per cent. The milk acidity from the cows was throughout between .1 and .13 per cent. There is then a chance that some of the sugar in the material extracted from the udder was converted into acid before it could be read. Another and perhaps more serious chance to decrease the lactose accounted for in comparison with the milk of the 3 previous days is a reduced milk secretion on the day of killing incident to the trip to the slaughter house. Thus all the factors tend to reduce the milk accounted for in contrast to the average of the 3 previous milkings.

Our data are in general accord with those of Gaines and Sanmann. Their technique on Cow 2 is, we believe, better than ours in that there is less chance of losing lactose in the manipulation of the udder and we are inclined to the view that our results show somewhat less lactose than was actually present in the udders.

The available information thus indicates that the mammary gland at time of milking contains the majority of the lactose to be secreted in milk.

Relation between the Size of the Mammary Gland and the Milk It Secretes.

The relation which exists between the size of a gland and the size of the product which it manufactures is an almost unstudied problem of gland physiology. In fact the problem has been approached only by judges of dairy cattle. These qualitative data have led to the assertion that the size and quality of the udder does to some extent indicate the productive capacity of the cow. The records of these nine cows in the experiments herein described furnish unique, fairly exact evidence on this problem. The correlation which can be derived from these data is admittedly open to a very large probable error. It does, however, furnish an important guide to the results which might be expected on more extensive data. The material also has the advantage that the determinations are quantitative and relatively accurate as contrasted with those heretofore used. Statistically considered the results as treated are significant since $r = .96$, $t = 9.0$ and P for $N = 9 < .01$.

The most interesting comparison is that between the weight of the udder with the contained milk and the milk production which the cow normally gave. This measurement is also the most exact so far as the collection of the data is concerned. Table VI shows the relation of the milk yield to the weight of the udder and contained milk.

Table VI and Fig. 1 show that the average weight of the udder and its contained milk increases as the milk production which the cow is then giving increases. This increase amounts to about 1.2 pounds

TABLE VI.

Total Weight of Udder and Contained Milk Contrasted with the Cow's Average Milk Yield at the Same Period.

Cow No	Average milk yield	Weight of udder and contained milk
114	4 1	16 6
97	4 7	18 8
153	10 8	26 1
154	11 7	24 2
111	12 9	29 3
148	13 5	27 2
132	15 6	28 7
136	15 7	30 6
124	17 9	36 0

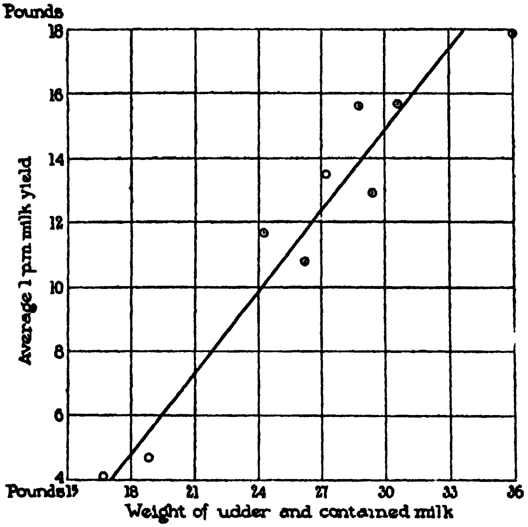

FIG. 1. Relation between average 1 p.m. milk yield and the weight of the udder and contained milk.

for the weight of the udder and its contained milk as the cow increases in milk production 1 pound, the increase being fairly regular over the entire range. From this fact it may be concluded, tentatively, as the probable errors are large, that for creating a pound of milk between 10.00 p.m. and 1.00 p.m. (15 hours), .2 of a pound of secreting mammary tissue would be necessary. On the basis of these few observations the relationship between milk yield and the mammary gland size appears to be linear. In view of this fact, if the line of general relationship is extended to the point of no milk production, the udder would weigh about 12 pounds. On the basis of the results for Cows 97, 114, and 153 such an udder would still contain 2 to 3 pounds of milk. The mass of udder tissue in the practically dry cow would consequently be 9 to 10 pounds. This might be conceived of as connective tissue supporting the glandular structure, furnishing the surrounding tissue for the teats and alveola spaces.

The Remaining Udder Material.

As indicated earlier the udder with its contained milk was first cut and drained of the milk which would flow out of it. The remaining material was then ground and extracted with water for three extractions, 50 pounds of water being used in all but one of the extractions where 40 pounds was used. After each extraction the udder material was placed in a cheese-cloth bag and allowed to drain for varying lengths of time. The udder material remaining after this treatment was then weighed. These weights are indicated in Table VII. A large part of this weight was water replacing the soluble materials washed from the udder. This remaining udder material was dried to constant weight and extracted with gasoline until all the fat was removed. It then was dried and ground and the percentage of remaining lactose determined. The determination of the percentage of water and fat removed from the remaining udder material is shown in Table VII.

Column 2 of Table VII shows the remaining udder material after it has been washed by the three extractions. It will be noticed that the amount of this material has a fairly close correlation to the yield of milk which the cows are giving.

The percentage of water and fat which was found in this material is shown in the third column. This water and fat varies between 85 and 91 per cent of the udder material. As would be expected from the fact that the length of time in draining the udder was not constant for each cow, this percentage variation is quite random.

The weight of the remaining udder substance after the extraction of water and fat is shown in the fourth column of the table. The amount of udder substance varies between 1.4 and 2.3 pounds. On the basis of the total weight of the udder and its contained milk this dried material, gasoline- and water-soluble-free, is 5 to 10 per cent of the udder weight.

If the weight of the remaining udder substance is compared with the yield of milk which the cow is able to give, but little correlation is found. This lack of correlation is quite likely caused by the fact that these cows were, in general, producers of about the same capacity. The lower productions of 97 and 114 represent the drying off of these cows. This insoluble udder substance may be regarded as largely supporting tissue for the secreting cells and therefore of a more or less permanent nature. By the method of treatment this material appears to be of about the same weight for the cows secreting milk and those which are approaching the dry period.

TABLE VII.

Remaining Udder Material after Extraction, and Percentage of Water and Fat Removed from the Same.

Cow No.	Remaining udder material	Percentage of water and fat	Weight of remaining substance	Milk yield total
111	15.7	87.7	1.93	20.3
124	16.9	89.6	1.76	27.9
132	14.5	87.3	1.84	25.9
136	16.5	86.1	2.29	29.5
148	15.5	90.8	1.43	21.8
154	15.2	89.3	1.63	17.7
97	12.0	84.7	1.84	6.8
114	11.0	87.4	1.39	6.9
153	14.5	89.6	1.51	17.8

SUMMARY.

The results herein presented furnish exact critical evidence for the conclusion that the most of the milk is present as such in the udder of dairy cattle at the time of milking. The amount of milk which may be secreted during milking cannot, on the basis of these results, be over 20 per cent of the milk yield of the cow.

The results show clearly that the size of the udder measures closely the amount of milk which the cow is able to secrete.

The results indicate that about 1/5 of a pound of secreting tissue is necessary for the secretion of a pound of milk during a period of 15 hours. The weight of the udder during the period that the cow is dry appears to be between 6 and 8 pounds.

THE EFFECT OF TEMPERATURE UPON SOME OF THE PROPERTIES OF CASEIN.

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I.

INTRODUCTORY.

The considerable changes which occur in the behavior of living matter when subjected to high or low temperatures raise interesting questions as to how the temperature affects the behavior of certain substances present in nature.

Casein, purified at the point of its maximum flocculation, is able to bind base, forming a salt soluble in water. The amount of casein which passes into solution is not only dependent upon the amount of base added, but also upon the temperature.

We propose to study the interdependence of three variables: the base added, the casein dissolved, and the temperature.

The properties of casein when dissolved in alkali and combined with base have been studied by a large number of investigators and by different methods. The results obtained may conveniently be considered in terms of the different equivalent combining weights for base that they have ascribed to casein.

II.

The Equivalent Weight of Casein.

The combining weight of casein was investigated before the development of the electrolytic dissociation theory. In 1865, Millon and Commaille (1, 2) stated: "...la caséine, matière unique, s'unirait sans doute à la plupart des acides minéraux et organiques, et si toutes ces combinaisons étaient réellement bien définies, il ressortirait de leur examen une connaissance plus éxacte de la caséine, de sa formule, de son équivalent et de son affinité."

Millon and Commaille assigned to this protein the formula weight of $C_{108}\ H_{97}\ Az_{14}\ O_{29}$ for its equivalent (1), which upon calculation yields 2050 gm. This number, as we shall see, is in excellent agreement with the equivalent weight of this protein obtained by modern workers. It seems plausible to give credit for the discovery of this important number in the chemistry of casein to these investigators, although this discovery was of a somewhat accidental nature.

TABLE I.

The Titration of Casein with Base.

Investigator (1)	Year (2)	Method (3)	Equivalent of base $\times 10^{-4}$ bound by 1 gm. casein (4)	Gm. of casein bound by 1 equivalent of base (5)
Söldner (4)	1888	To phenolphthalein	8.16	1230
Courant (5)	1891	“ “	9.5	1050
Timpe (6)	1893	“ “	9.35	1070
Laqueur and Sackur (8)	1903	“ “	8.80	1135
Matthaiopoulos (9)	1908	“ “	8.85	1130
Hart (10)	1909	“ “	9.25	1080
Pfyl and Turnau (11)	1914	“ “	8.75	1145
Peroff (12)	1921	“ “	8.20	1220
Bleyer and Seidl (13)	1922	“ “	8.77	1144
Average				1124
Robertson (15)	1910	Electrometrically at pH 8.50	8.0	1250
Loeb (14)	1921	“ “ “ 8.50	6.6	1520
Cohn (23)	1925	“ “ “ 8.50	6.3	1590
Robertson (15)	1910	“ “ “ 7.00	5.1	1960
Loeb (14)	1921	“ “ “ 7.00	4.3	2340
Cohn (23)	1925	“ “ “ 7.00	5.0	2000

Hammarsten (3), in 1877, greatly improved the method of preparation of casein, which with few modifications is still used by modern investigators. He (3) also discovered that casein decomposes $CaCO_3$ liberating CO_2, which indicated that this protein is an acid.

Titration of Casein to Indicators.—Many investigators have studied the acid properties of casein, titrating it with base to a suitable indicator. Phenolphthalein was chosen most often, as the appropriate method of titrating a weak acid, casein, and a strong base.

Söldner (4) found that 8.3 cc. of 0.10 N mono- or divalent base were required to bring 1 gm. of casein to neutrality to phenolphthalein. Since Söldner's research, Courant (5), Timpe (6), Bechamp (7), Laqueur and Sackur (8), Matthaiopoulos (9), Hart (10), Pfyl and Turnau (11), Peroff (12) and Bleyer and Seidl (13) have titrated casein to indicators. Most of the results of these investigations are recorded in Table I. On the whole, these data agree fairly well with each other, showing the existence of a definite chemical substance, endowed with a certain base-binding capacity. Loeb (14) showed that the bases combined with casein in equivalent proportions whatever the end-point to which this protein was titrated.

Electrometric Studies upon Casein.—From our present knowledge of the chemistry of casein, the amount of base bound to phenolphthalein is of no fundamental importance; it is merely a single point on the titration curve of casein. Casein can bind far more base in more alkaline solutions. In 1910, Robertson (15) found that the casein preparations that he studied bound, at saturation, as much as 180×10^{-5} equivalents of base per gm. of casein.

Later electrometric estimates of the maximum base-combining capacity of various casein preparations (15–18) showed the existence of at least two modifications of casein: one binding about 180×10^{-5} equivalents of base per gm. and having a steeper titration curve and the other termed "unmodified" casein, having a maximum base-combining capacity of 138×10^{-5} equivalents and a flatter titration curve. The former of these modifications appears when a casein preparation, during the course of its purification, is subjected to an alkaline[1] treatment (18).

Solubility Studies upon Casein.—Robertson (19) studied the amount of base necessary to hold casein in solution. He found that 1.14×10^{-4} equivalents of monovalent base is sufficient to hold 1 gm.

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[1] For a review of the subject, as well as the shape of the titration curves of caseins, consult Cohn, E. J., The physical chemistry of the proteins, *Physiol. Rev.*, 1925, v, 349.

of casein in solution (15). In systems containing $Ca(OH)_2$, the amount of base bound by casein was found to be 1.19×10^{-4} equivalents (20).

L. L. Van Slyke and Bosworth (21), using a very similar method, found that 1 gm. of casein forms a soluble compound with 1.10–1.115

TABLE II.

The Equivalent Weight of Casein.

Investigator (1)	Year (2)	Method (3)	Equivalent of base $\times 10^{-4}$ bound by 1 gm. casein (4)	Gm. of casein bound by 1 equivalent of base (5)
Robertson (19)	1909	“Saturation” monovalent bases	1.14	8800
Van Slyke and Bosworth (21)	1913	“ “	1.125	8890
“ “ “ “ “	1913	“Saturation” divalent bases	2.25	4440
Millon and Commaille (1, 2)	1865	Analysis	4.88 and more	2050 and less
Long (24)	1907	Solubility	4.50	2220
Yamakami (22)	1920	“	(2.4)	(4.150)
Cohn and Hendry (25)	1923	“	4.76	2100
Greenberg and Schmidt (16)	1924	Electrochemical equivalent	4.96	2010
Carpenter (28)	1926	Formol titration	4.65	2140
Average of last six				2104
Cohn and Berggren (18)	1925	Maximum base-binding (“unmodified”)	13.8	725
Robertson (15)	1910	Maximum base-binding (“Nach Hammarsten”)	18.0	550
Cohn and Berggren (18)	1925	“ “	18.3	545

$\times 10^{-4}$ equivalents of monovalent bases. Dialyzing the solutions of casein with divalent bases, they found that the amounts of these bases necessary to hold casein in solution were just twice the amounts of monovalent bases. In Table II we are referring to the method used by Robertson and by Van Slyke and Bosworth as the “saturation” method.

Yamakami (22) adding NaOH to casein and, filtering off the undissolved casein, found that 2.25 to 2.50 $\times$ 10^{-4} equivalents of base dissolve 1 gm. of casein. This upon calculation yielded 4.150 gm. for the equivalent weight of casein, that is the weight of casein bound by 1 gm. equivalent of base.

Long (24), in 1907, found that 5 gm. of casein will dissolve completely in 22.5 cc. of 0.1 N alkali, which upon calculation yields 2220 gm. for the equivalent weight of casein, based on these solubility measurements.

The solubility of casein in NaOH solutions was extensively studied by E. J. Cohn and Hendry (25). From their measurements they found the equivalent weight of casein to be 2100 gm. These investigators came to the important conclusion that the only determining factor in the solubility of casein with NaOH is the amount of base added. The amount of casein may be varied without affecting the solubility.[2] This value of the equivalent weight of casein has been recently confirmed by various methods. Greenberg and Schmidt (27), from the study of the electrochemical equivalent of casein, calculated the equivalent weight as 2015 gm. Carpenter (28), using the formol titration method, concluded "that one formula weight of amino-group is linked with carboxyl per 2150 gm. of casein."

Certain relationships exist between the equivalent weights obtained by various investigators from their solubility measurements. The equivalent combining weight with monovalent bases determined by the so called saturation method, 8800 to 8890 is just twice the weight obtained by the same authors with divalent bases, 4440. Half of this value, 2220, yields the equivalent combining weight determined by solubility, by transport number, or by formol titration. Again, one-third of the equivalent weight of casein determined by these methods is equal to the maximum base-combining capacity of "unmodified" casein, 725, and one-fourth to that of casein modified by alkaline treatment, 545. The researches carried out by many investigators thus reveal the existence of certain definite compounds of casein that exist under specified conditions.

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[2] Compare with Linderstrøm-Lang's investigation (26) upon the solubility of casein with acids.

III.

EXPERIMENTAL.

Casein Preparation.—The proteins used in this investigation were prepared according to the modified (25) method of Van Slyke and Baker (29). The caseins were prepared from fresh unpasteurized milk. They were precipitated by the addition of HCl, and redissolved with NaOH. From fear of modification, we have never exposed our caseins to a hydrogen ion concentration less then pH 7.00. The casein solutions were passed through a Sharples centrifuge, filtered through paper pulp filter, and reprecipitated with HCl. This procedure, resolution, and reprecipitation, was repeated once or twice. Finally, the casein precipitate was extensively washed with distilled water until chloride-free. The procedure for the preparation of casein has already been reported in detail (25).

The Measurement of Solubility.—The solubility measurements were carried out very much as described in a previous communication from this laboratory (25). Samples of uncombined casein precipitates were placed in 100 cc. volumetric Pyrex flasks and the required amount of very dilute NaOH was slowly added. Next, the flasks were filled to the mark with CO_2-free distilled water and placed in a shaking machine for equilibration. All the reagents were always used at the temperature at which the experiment was set.

Two shaking machines were used in the process of equilibration of the protein with NaOH. One, less efficient, was used in the experiments at 25° and at 37°C. The other, at least three times more efficient than the former, was used for all experiments at other temperatures. The times for equilibration given in the tables describing our experiments are therefore not strictly comparable to each other.

After the systems containing casein had reached equilibrium, they were filtered through No. 42 Whatman's paper filters. The filtration was carried out at the temperature of the experiment, except in those done at 49°. In this investigation, the filtration was carried out at about 20°C. As we shall see in a subsequent section of this paper, this difference of temperature did not interfere with the measurements.

Aliquot parts of the filtrates were then analyzed for nitrogen by the Kjeldahl method. Allowance was made in calculations for the small amounts of nitrogen present in the reagents. The analyses were carried out in triplicate. In all calculations we used the factor 6.40 for converting the amount of casein nitrogen into the amount of the protein,—that is, the per cent of nitrogen in casein was assumed to be 15.62.

If one calculates the results of the analyses on the basis of the solution, without taking into consideration the precipitate, the result will be a figure slightly higher than the true one, as the total volume of a suspension is made up of two parts: the volume of the solution and the volume of the precipitate.

In all our experiments the amount of casein in the form of a precipitate was less than 1 per cent, and since the density of casein precipitate is about 1.26 (30) the error is less than 1 per cent and can be neglected for the present.

IV.

The Effect of Temperature on the Solubility of Casein in Water.

The solubility of casein in water at the point of minimum solubility has been the subject of several investigations. Bechamp (7) found that freshly precipitated casein, free from acid, was soluble in water. According to this investigator, the solubility is dependent both upon time and temperature. From 0.24 to 1.005 gm. of casein was found to dissolve in 1 liter of water between the temperature range of 15° and 25°C. In order to obtain the maximum solubility, Bechamp found it necessary to triturate casein with water for 52 hours. Under these conditions, at 20°C., casein was found to be soluble in water to an extent of 1.005 gm. per liter of solution.

Osborne (31), Laqueur and Sackur (8), and Robertson (32) believed that no appreciable amount of casein dissolved in water. Yamakami (22) triturated casein with distilled water and after leaving the suspension standing for a considerable period of time at low temperature, found from 0.0118 to 0.0156 gm. of casein per 100 cc. of the filtrate. He, however, thought that the reason for this phenomenon was purely manipulative.

Cohn (33), after a prolonged agitation of suspension of casein in water, found that this protein dissolves to a constant and characteristic amount, the amount dissolved being independent of the amount of the casein in the suspension; 0.11 gm. of casein according to this investigator, is dissolved in 1 liter of water at 25.0°C.

As we see, the results of these investigations disagree widely. It is quite likely that the earlier measurements of the solubility of uncombined casein in water are incorrect. Accurate measurements became possible after the development of the modern knowledge of the isoelectric points of proteins.

It was of interest to investigate the effect of temperature upon the solubility of this protein in water as compared to the effect of this factor on the capacity of casein to bind base. If these two phenomena are unequally affected by the temperature, they are likely to be of different physicochemical nature. If, conversely, they are equally increased or decreased by the temperature, they probably are functions of a single physicochemical property of the protein.

The results of our investigation on the solubility of several preparations at 5.0°C. and of Casein Preparation XXVII at 25°C. are reported in Table III.

From the results of these measurements the solubility of uncombined casein in water at 5.0°C. may be taken as being equal to about 0.70 ± 0.10 mg. of N per 100 gm. of water, or, in terms of the protein, 0.045 gm. are dissolved per 1000 gm. of water.

Table III also includes an investigation of the solubility of Casein Preparation XXVII at 25°C. It appears from our experiments that this casein preparation dissolved to a slightly greater extent than the value given by Cohn (33) for the solubility of this protein in water. It seems advisable, therefore, to widen the limits of the solubility of casein in water, assigning to this solubility 2.00 ± 0.30 mg.

of nitrogen per 100 gm. of water at 25°C., or, in terms of casein, about 0.13 gm. of casein are soluble per 1000 gm. of water.

We also attempted to measure the solubility of casein in water at 37°C., but found that the solubility at this temperature, unlike that at 25°, was dependent upon time, indicating, presumably, a progressive hydrolysis of the dissolved casein.

From the data at hand, we may conclude that the solubility of casein in water is dependent on the temperature: an increase of 20°C. nearly triples the solubility of this protein in water.

A comparison of the increase of the solubility in water of casein with the corresponding change in the capacity of casein to bind base (Fig. 2) indicates that these phenomena quantitatively are not identical: the change in the acid properties of casein, as measured by its solubility in base, is less than the change in the solubility of this protein in water, which suggests that these phenomena may depend upon independent chemical reactions.

TABLE III.

The Solubility of Casein in Water at Various Temperatures.

Temperature °C. (1)	Casein preparation (2)	Experiment No. (3)	Time for equilibration (4)	Solubility: Mg. N dissolved per 100 gm. of H_2O (5)
			hrs.	
5.0±0.5	XXIV	26	45	0.80
	XXV	25	45	0.60
	XXVII	29	40	0.64
	"	34	20	(0.40)
25.0±0.1	"	32	70	2.35
	"	31	62	2.30
	"	41	23	2.20

V.

The Effect of Temperature upon the Solubility of Casein in Base.

In 1888, Söldner (34) noted that the salts of casein with Ca, Ba, Sr, and Mg become opalescent when heated to about 40°C. The opalescense disappeared upon the cooling of the solution. Osborne (31) confirmed these observations and also found that Li caseinate shows a very slight turbidity when warmed; ammonium caseinate, however, showed no sign of precipitation. Osborne explained this phenomenon in terms of hydrolysis of the protein salt: on heating the solution, casein is formed, which being insoluble, produces the opalescense. In 1908, in an extensive investigation upon the effect of temperature upon the solubility of cases with bases, Robertson (35) found that, unlike the systems containing casein and bivalent bases, those containing this protein and monovalent bases are affected by the temperature in the opposite manner: with the rise in temperature the solubility increases. Robertson correctly pointed out that the hypothesis of Osborne did not explain the effect of temperature upon the solubility of casein in monovalent bases.

TABLE IV.

The Solubility of Casein in Alkali at Various Temperatures.
Robertson (35).

Amount of base added equivalent $\times 10^{-5}$	Solubility: Gm. of casein dissolved per 100 cc. at							
	21°	36°	46°	54°	60°	66°	81°	88°
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
KOH								
23.0	0.46					0.52		
46.0	0.92	0.92	1.04		1.34	1.27	1.34	1.13
69.0	1.38					2.11		
92.0	1.85			2.77		3.05		
LiOH								
44.0	0.89	0.86			1.28	1.14	1.60	1.20
88.0	1.77			2.62		2.95		
$Ca(OH)_2$								
45.0	0.90	0.72			0.65	0.63	0.64	0.63
90.0	1.80			1.35				

Robertson (35) concluded that:

> "the facts are much more readily explained on the supposition that the effect of temperature consists in shifting the equilibrium,
>
> $$HXOH + HXOH \rightleftharpoons HXXOH + H_2O$$
>
> toward the right so that a given amount of alkali (since it is associated with a molecule of nearly double that weight) neutralizes nearly twice as much casein

at 66° as it does at room temperature (21°). The marked diminution in the solubility of casein in $Ca(OH)_2$ solutions, which occurs on raising the temperature, can be explained by supposing that the salt $Ca(XXOH)_2$ is insoluble, while the salt $Ca(XOH)_2$ is soluble."

However, Robertson's theory, essentially based on the supposition that the protein's ultimate particle could be easily broken into two, has met with considerable criticism. Its application to the ionization of the proteins was unquestionably disproved by Greenberg and Schmidt, who, in a series of experimental studies (27, 36) upon casein, have shown that the behavior of this protein can be explained upon the assumption of the formation of a single protein ion.

Furthermore, Robertson's own data (Table IV) do not favor his theory: an increase of temperature from 21° to 60° does not double the solubility of casein in base and the amount of protein dissolved at 60° can hardly be increased by a further rise of temperature. The phenomenon seems to be of a far more complex nature.

Although we disagree with some of the aspects of Robertson's interpretations of his experiment, the data themselves, as we shall see, are in excellent agreement with recent solubility measurements upon casein. Since in a further discussion of this subject we shall extensively use Robertson's data, we shall first reproduce his table (Table IV), replacing the "concentrations of base" by the "amount of base added," and secondly, give a short description of his experimental procedure.

A given amount of base (35), diluted to 100 cc. with distilled water, was warmed to the desired temperature in a thermostat, which was kept constant within 0.5°. Then three times the amount of casein which would be dissolved by the given amount of alkali at room temperature (*i.e.* 3 gm. to every 5 cc. N/10 alkali) was introduced and the mixture left in the thermostat for from 30 to 40 minutes, being vigorously shaken at frequent intervals. The resulting solution was then filtered at the same temperature and the filtrate allowed to cool.

The estimate of the amount of casein dissolved was made by titrating the solution to phenolphthalein. Knowing that 0.125 gm. of casein bind 1 cc. of 0.1 N alkali to this indicator, and the amount of alkali already added to the sample, Robertson was able to estimate the amount of casein dissolved.

TABLE V.

The Solubility of Casein in NaOH at Various Temperatures.

Temperature °C. (1)	Casein preparation (2)	Experiment No. (3)	Time for equilibration (4)	Mols NaOH $\times 10^{-5}$ added (5)	Solubility: Mg. casein N dissolved per 100 gm. H_2O (6)
			hrs.		
5.0±0.5	XXVII	42	42	0.50	1.05
	"	"	"	"	"
	"	33	18	1.00	2.75
	"	42	42	"	2.30
	XXV	19	4	2.00	5.1
	XXVII	33	18	"	5.50
	XXV	23	25	"	5.25
	XXVII	42	42	"	5.50
	XXV	19	4	4.00	8.87
	XXVII	33	18	"	9.03
	XXV	23	25	"	8.45
	XXV	19	4	6.00	12.8
	XXVII	33	18	"	12.9
	XXV	23	25	"	13.1
37.0±0.5	XXVII	35	45	2.00	8.95
		36	50	"	9.60
	"	35	45	4.00	16.2
	"	36	50	"	15.7
		38	100	"	(17.9)
	"	35	45	6.00	22.7
		36	50	"	22.4
49.0±0.3	"	43	2	10.00	29.8
		"	4	"	29.5
	"	"	2	20.00	71.8
	"	"	"	30.00	111
	"	"	4	"	114

The casein solutions subjected to such high temperature as 88°C., used in certain of Robertson's experiments, are indeed likely to undergo a hydrolytic scission. Robertson (35) found that 46×10^{-5} equivalents of KOH at 88° after half an hour dissolved 1.13 gm. of casein. The same solution kept for 3 hours dissolved 1.25 gm. The error, according to Robertson, would be about 0.04 gm. in half an hour at 88°.

At lower temperatures, the error must be still smaller, and at 49° as our measurements show (Table V), increasing the time for equilibration from 2 to 4 hours hardly changed the solubility of casein in NaOH.

It is therefore quite probable that up to 80°C. the hydrolysis of casein does not appreciably affect the solubility measurements, provided of course the solutions are not exposed to high temperature for a long period of time.

The results of Robertson's investigation at 21° and at 66° are reproduced in Fig. 1. For comparison, on the same chart are plotted the results obtained by Cohn and Hendry (25) with NaOH at 25°. It is evident from this graph that the results of Robertson's measurements yield an excellent straight line. The equivalent weight of casein, calculated from these experiments, is 2000 gm. at 21°C. Furthermore, KOH, LiOH, and Ca(OH) dissolve casein at this temperature in equivalent proportions.[3]

The equivalent weight calculated by us from Robertson's experiments is in close agreement with the one obtained by Cohn and Hendry. The former is 2000; the later 2100. The agreement is within about 5 per cent. The comparison is possible because (Table IV, Fig. 4) the solubility of casein with monovalent bases in this range is independent of the temperature, the combining weight remaining constant throughout the range from about 21° to 37°.

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[3] Robertson formerly held the same opinion. Later, however, he gave it up in favor of the evidence brought about by the experiments of Van Slyke and Bosworth (21). The statement that casein is dissolved by $Ca(OH)_2$ in equivalent proportions is not strictly correct. As our unpublished measurements indicate the relationship obtained by Robertson holds true only under certain experimental conditions. Unlike the solubility with monovalent base the solubility of casein with divalent base is a function of the amount of casein in the system. Such systems will be considered in a separate communication.

It is of interest to consider these two sets of experiments from the point of view of the equilibrium condition. The time of equilibration in Robertson's experiments was about 35 minutes. The corresponding time in Cohn and Hendry's experiments varied from 24 to 72 hours (25). It is evident that a 150 fold variation of the time of equilibration had practically no effect upon the solubility of the casein. We may

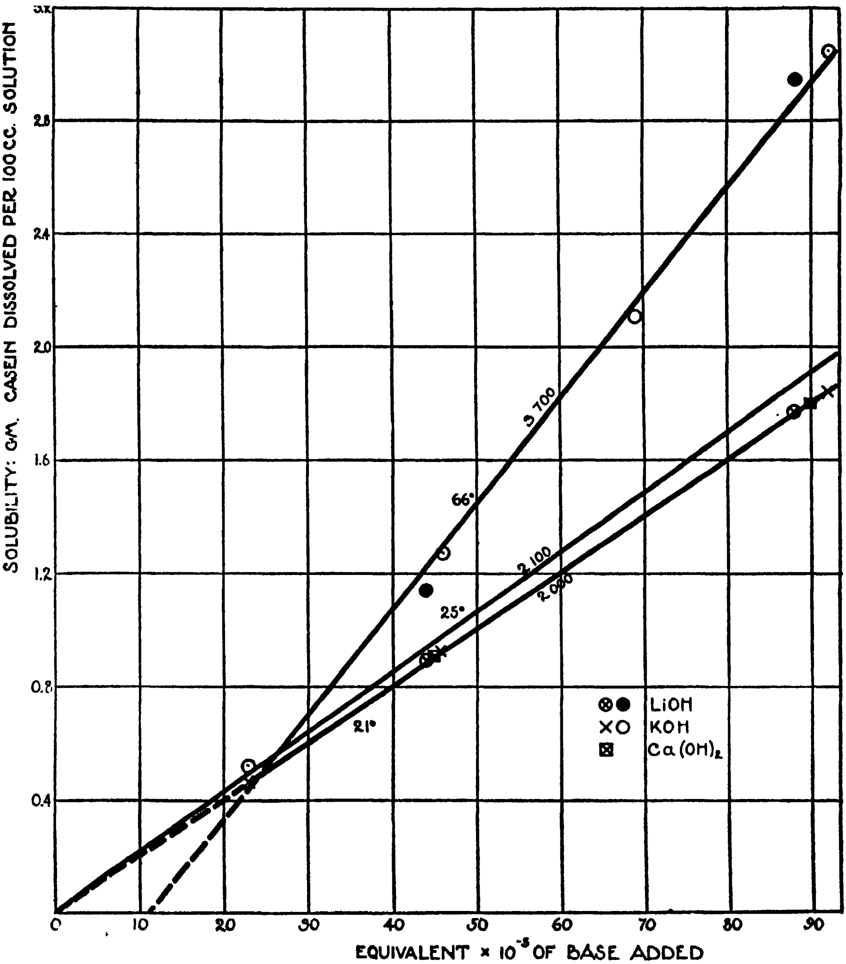

FIG. 1. The solubility of casein in bases at 21° and 60°C. according to Robertson (35) and at 25°C. according to Cohn and Hendry (25).

conclude from this that, first, casein is readily soluble in alkali; and, second, that soon a stable state is reached, the solubility of the casein being practically independent of time. The latter is a criterion for the equilibrium conditions.

There is one more conclusion that can be obtained from the comparison of these two investigations. Robertson estimated the amount of casein dissolved by the amount of base bound by this protein in solution; Cohn and Hendry estimated the same quantity directly, by estimating the amount of casein in the liquid phase. The equivalent weights correspond graphically to the slopes of the lines relating the amount of base added, and the amount of casein in solution. The slopes of these lines in these two cases agree within 5 per cent, which evidently means that casein dissolves and binds base at the same rate.

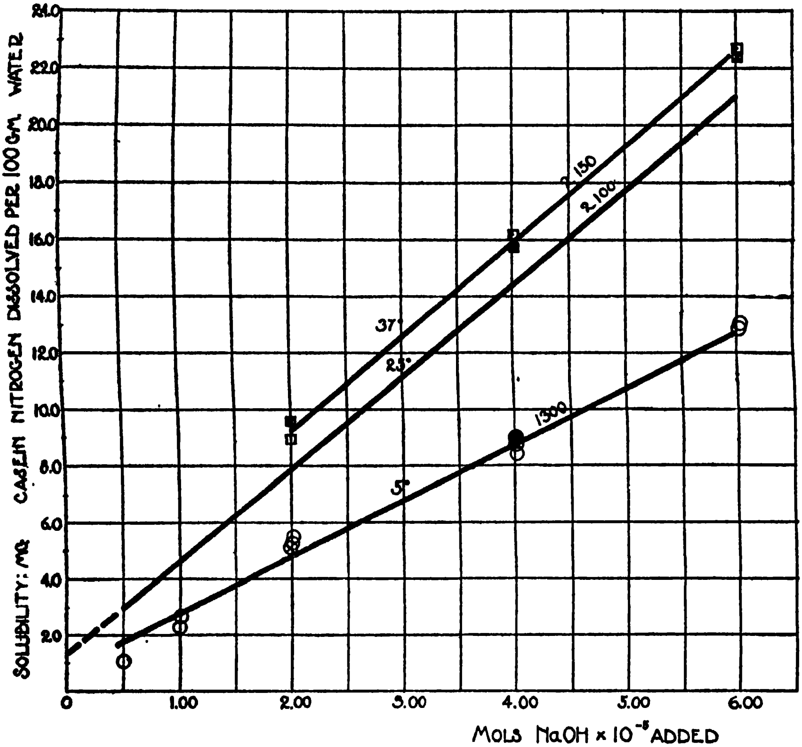

FIG. 2. The solubility of casein at 5°, 25°, and 37°C. in small amounts of NaOH.

To extend and supplement the observation of Robertson, we have carried out measurements of the solubility of casein in NaOH at various temperatures. These results are reported in Table V. They are graphically represented in Figs. 2 and 3, together with the calculated equivalent weights in gm. of casein. Cohn and Hendry's solubility measurements at 25°C. are also given on the same chart, for comparison.

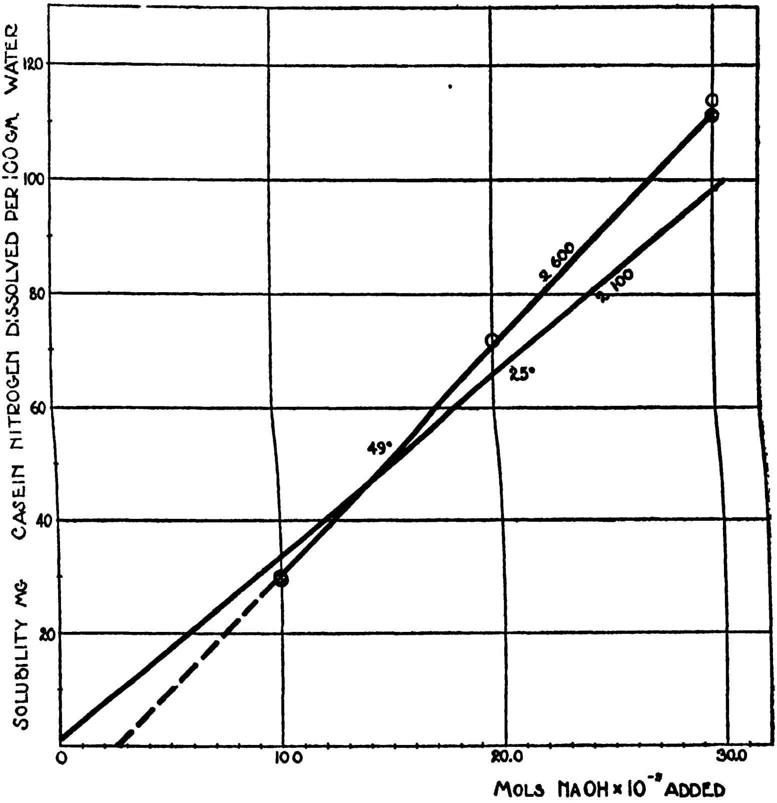

FIG. 3. The solubility of casein in NaOH at 25° and at 49°C.

For most of the experiments, Casein Preparation XXVII was used. This protein dissolved at 25.0°C. to the extent of 2100± 50 gm. per 1 mol of NaOH.

The time of equilibration for our individual experiments was recorded. At 5.0°C. we greatly varied the time of equilibration, since at low temperature we suspected a slower rate of reaction. As it appears from Table V, about 4 hours of continuous shaking is sufficient to bring this system to rest. The variation between individual measurements is within the experimental error. At 37°C. it appears that up to 50 hours of equilibration the solubility is not a function of time. However, doubling the time of equilibration produces an increase in the solubility outside the experimental error. The reason for this is probably an incipient hydrolysis. For this reason, the last figure was omitted from the calculations.

TABLE VI.

The Equivalent Weight of Casein from Solubility Measurements at Various Temperatures.

Investigator (1)	Temperature °C. (2)	Equivalent weight (3)	Remarks (4)
		gm.	
Author	5.0	1300	With small amounts of base
Robertson (35)	21.0	2000	
Cohn and Hendry (25)	25.0	2100	Average: 2070
Author	37.0	2150	
Author	49.0	2600	
Robertson (35)	66.0	3700	From 60° to 85° the equivalent weight again remains practically constant

The solubility of casein in small amounts of NaOH at 5°C. is distinctly different from its solubility at 25°C. (Fig. 2). In the range investigated, about 1300 gm. of casein are carried into solution by 1 mol of NaOH while according to Cohn and Hendry (25), 2100 gm. pass into solution per 1 mol of NaOH added, in this range at 25°C. The solubility of casein in small amounts of NaOH at 5°C. is unaffected by the amount of the saturating body. We have varied the amounts of this protein without appreciably changing the solubility. When casein begins to dissolve at 5°C., the slope of the solubility line may be, therefore, taken as being equally characteristic of this protein, as is the solubility of casein at 25°C.

In a separate communication we shall take up further consideration of this case in relation to the solubility of this protein in $Ca(OH)_2$.

In Table VI, we have compiled the value of the equivalent weights with monovalent bases at various temperatures.

Outside these data, we have the single determinations of Robertson (Table IV) from which there is no way to estimate directly the equivalent weight, since the origin of the solubility lines (Figs. 1, 2, and 3) varies with temperature and probably with the method used. These points cover the range from 21° to 60°C., as well as the temperatures higher than 66°C.

From these data the equivalent weights were calculated upon the following considerations. First, as we may note from Table IV, the solubility of casein in monovalent bases is probably constant in the range of 21° to 36°C. The origin of the line (Fig. 1) at 21° C. as far as we can estimate it, is at the zero point. Since no change in the solubility occurred, up to 36°C., it is plausible to assume that the origin of the line at this temperature has not changed.

A similar consideration holds true for the range of temperature from 60° to 80°C. The scattering of the experimental points is greater than at 21° (see Fig. 1). They do not show any decided tendency either to increase or to decrease. We have assumed therefore that the origins of the lines of the solubility measurements have not appreciably changed. The line at 66° C. (Fig. 1) cuts the abcissa at about 11.0×10^{-5} mols of base.[4]

It is therefore probable that the change of origin of the lines relating the solubility with the base added takes place at the temperatures from about 37° to 60°. The equation for this change as a function of temperature is not available. We have assumed it to be a straight line, and from this line calculated the intermediary corrections listed in column (3) of Table VII. Even if this relation in Robertson's experiments is not a straight line, the diversion from the straight line will only be a fraction of the correction while the correction itself is only a fraction of the amount of base added.

It is evident that the exact shape of the line is of no great importance to the calculated result.

Using these corrections for the origins, we have calculated in Table VII the equivalent weights for the single observations of Robertson.

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[4] Throughout this investigation we often refer to the origin of the solubility lines. This reference is made purely for the evaluation of the data. No physical significance should be attached to the values obtained by extrapolation, since it is always possible that with small amounts of base at high temperature casein would display a different combining weight, which might bring the origin nearer to the zero point. We have tried to measure the solubility of casein in small amounts of base at high temperature but found that these solutions underwent a rapid hydrolysis.

They are reproduced in Fig. 4, with the data compiled in Table VI. As we see, they agree satisfactorily with our own determination at 49°C.

If, however, this method of procedure seems not plausible enough, the conclusions obtained in the next section of this report can be deduced without the calculations of Table VII.

These calculations, as we believe, complete the quantitative formulation of the effect of temperature upon the properties of casein, and in this sense have their place.

From the physicochemical standpoint, it is of considerable interest to learn whether changes brought about by the temperature in the capacity of casein to bind base are reversible.

TABLE VII.

Calculation of the Equivalent Weight of Casein from Robertson's Experiments.

Temperature °C. (1)	Mols × 10^{-5} of base added (2)		Correction: Mols × 10^{-5} of base to be subtracted (3)	Amount of LiOH or KOH added (corrected) Mols × 10^{-5} (2) − (3) (4)	Casein dissolved (5)	Equivalent weight (5) + (4) (6)
	KOH	LiOH			*gm.*	*gm.*
36	46.0		None	46.0	0.92	2000
36		44.0	None	44 0	0.86	1950
46	46.0		4.6	41.4	1.04	2500
54	92.0		8.2	83.8	2.77	3300
54		88.0	8.2	79.8	2.68	3350
60	46.0		11.0	35.0	1.34	3800
60		44.0	11.0	33.0	1.28	(3900)

While Ca caseinates become opalescent on heating, and the opalescence disappears on cooling, no such evidence is visible in the case of salts of potassium or sodium. In order to test the reversibility of this system, we brought some of the suspensions used in the experiment at 49°C. to a temperature of 5°C., at which temperature they were continuously shaken for about 200 hours. Then the suspensions were filtered and the amount of casein in solution determined as before.

No evidence of any decrease in the solubility of casein was noted. It is difficult to conclude from this that the reaction is irreversible, because it is always possible that the period of shaking of the suspensions was not sufficient.

From an entirely different source we have evidence that no permanent change occurs in casein, when this protein is subjected to a moderately high temperature. During the course of the preparation of casein, as a rule, the addition of acid or base was made at a temperature of 5°C; while the centrifugation and filtration was in most cases (including Casein XXVII) carried out at room temperature (about 20°C.). If the increase in the equivalent weights up to 2000 at about 20°C. is a change of an irreversible nature, evidently we would not be able to obtain a casein displaying a lesser equivalent weight at a lower

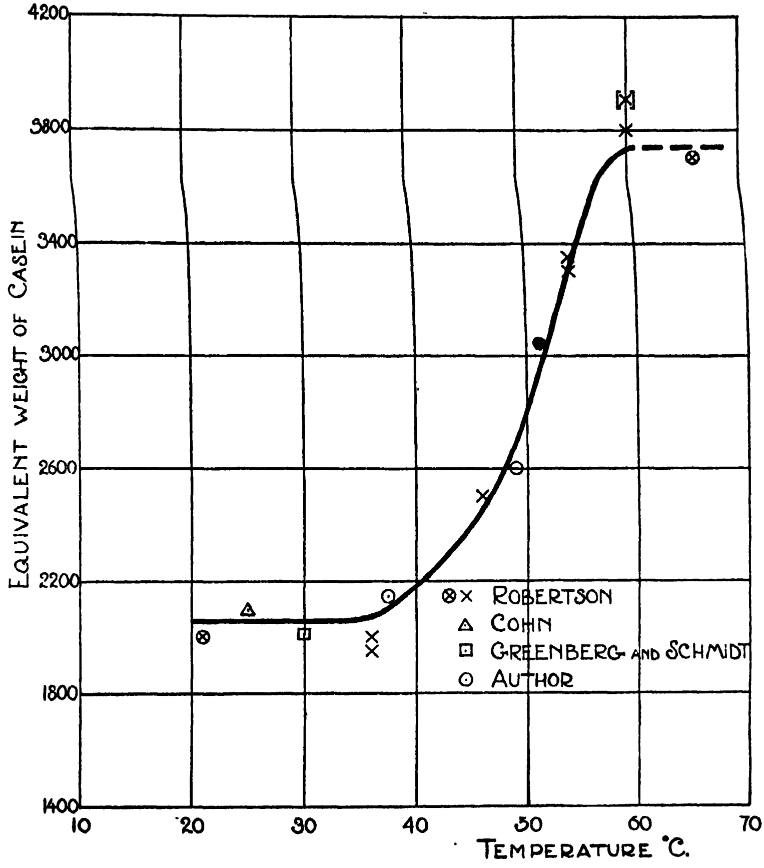

FIG. 4. The effect of temperature upon the equivalent combining weight of casein.

temperature. This is contrary to fact: the solubility of casein at 5° was found to be as low as 1300 gm. in some parts of the solubility curve. Therefore we must conclude that no permanent change occurs in casein, at least within the range of temperature from 5° to 20°C.

It is of considerable interest to extend such a conclusion to casein which was treated by base at a higher temperature. For this purpose one of the samples upon which the measurements of solubility with NaOH at 49°C. was carried out, and in which most of the protein was dissolved, was reprecipitated by addition of dilute HCl and washed free from the salt formed. Then to this precipitate 6.00×10^{-5} mols of NaOH were added and the mixture equilibrated at 25°C. This amount of NaOH dissolved 19.6 mg. of casein N, which, after correcting for the origin, yielded 1950 gm. for the equivalent weight of this casein.

Taking into consideration certain difficulties in manipulation with such small amounts of precipitate, we may conclude that the divergence from the average equivalent weight is not large enough to infer that a change occurred in the properties of the casein. The chemical cycle just considered may be outlined as follows:

$$\begin{aligned}
&\text{I. Uncombined casein} + \text{NaOH} = S_1 \xrightarrow{\text{heat}} S_2 \xrightarrow{\text{cool}} S_2 \xrightarrow[-\text{NaCl}]{} \\
&\quad \rightarrow \text{NaH caseinate} + \text{HCl} = \text{H}_2\ \text{casein} + \text{NaCl} \longrightarrow \\
&\rightarrow \text{II. Uncombined casein} + \text{NaOH} = S_1
\end{aligned}$$

I and II as far as we can judge are quantitatively identical equations.

V.

DISCUSSION.

The Solubility of Casein in Monovalent Base in a Range of Temperature from 21° to 85°C.—Upon inspection of Tables IV, VI, and Fig. 5, the following seems to be evident. First, the base-binding capacity of a casein system, as measured by the solubility, is not a continuous function of temperature. The equivalent weight remains constant from 21° to 37°C., having the best representative value, in round figures, of 2100 gm. From 37°C. to about 60°C. the solubility again becomes a function of temperature, and the equivalent weight rises from 2100 gm. to about 3700 gm. At a temperature higher than 60°C., casein with a rising temperature does not display any decided tendency either to increase its capacity to bind base, or to decrease it (Table IV). The scattering of the experimental points in the region of high temperature is greater than in the region of 21° to 37°C. A careful observation of the solubility of casein with KOH and $Ca(OH)_2$ (Table IV) favors this conclusion. The corresponding solubilities at 60°, 66°, and 81°C. from Table IV are 1.34, 1.27, and 1.34 gm. of casein. Upon inspection of Fig. 1, it is probable that the experimental point at 66° is slightly off of the average line. The corresponding point on the line is higher than 1.27, which brings these figures to a still better agreement in regard to their constancy.

A further justification of the existence of a plateau at the temperature higher than 60°C. is furnished by Robertson's experiments (Table IV) with $Ca(OH)_2$. It appears that the solubility of casein with $Ca(OH)_2$ is steadily decreased until the temperature of 60° is reached, after which the solubility remains remarkably constant.

It seems therefore plausible to conclude that at a temperature between 60° and about 85°C. there exists a region in which the solubility of casein is unaffected by temperature.

The numerical expression of the equivalent of casein at this plateau is probably 3700 gm. It is true that some of the single determinations show a slightly higher equivalent weight, but it must be remembered that from the statistical point of view the value obtained at 66°C., being based on several measurements, has a far greater weight than the single determinations at 60° and 81°, which furthermore, as we remember, were calculated by introducing a correction.

It seems reasonable, therefore, to conclude that the second plateau occurs when the equivalent weight of casein reaches the value of about 3700 gm.

In addition to the equivalent weight of casein determined by its solubility in monovalent bases, we can calculate, approximately, the amount of casein dissolved by one equivalent of $Ca(OH)_2$ at the temperature from 60° to 88°C. (Table IV). 1400 gm. of casein passes into solution per one equivalent of $Ca(OH)_2$ in this temperature range.

In a following communication we shall report an investigation upon the acid properties of paracasein. This modification of casein, according to our experiments, has the equivalent weight with NaOH of 1450 gm. at 23° ± 2°C.

The combining weights 3700, 2100, 1450, and 1400 have one characteristic in common. They are all estimates of the solubility of casein in bases at temperature levels at which the base-binding capacity of casein seems to be unaffected by changes of the temperature, within certain limits.

The discontinuous behavior of casein toward the temperature suggests that these levels may be associated with definite changes in the casein molecule.

In Table VIII is found an attempt to identify such a change. It appears that these combining weights are common multiples of 720, common multiples within a few per cent. The common factor, 720, in its turn (Table II) is very nearly equal to the maximum base-binding capacity of an "unmodified" casein, which is 725 gm. at 20° (18).

TABLE VIII.

An Application of the Law of Multiple Proportions to the Equivalent Weights of Casein at Various Temperatures.

Temperature range °C. (1)	Base used (2)	Equivalent weight (3)	Equivalent weight divided by 720 (3)/720 (4)	Whole numbers ascribed to (4) (5)
		gm.		
60-85	Monovalent	3700	5.1	5.0
21-37	"	2100	2.9	3.0
60-88	$Ca(OH)_2$	1400	1.95	2.0
21-25	Paracasein-NaOH	1450	2.0	2.0

The equivalent weight is a reciprocal measure of the acid or basic properties of a protein, since the greater the number of basic or acid groups involved, the smaller will be the weight of a protein combined with a given amount of acid or base.

It is therefore of interest for the estimate of acid properties of casein, to convert our combining weight into the amounts of base bound by 1 gm. of the protein. This has been done in column (4) of

Table IX. In column (5) of the same table we have calculated the ratio of the amount of base bound by 1 gm. of casein at saturation to the reciprocals of the remaining combining weights. The calculation yields a multiple relation, as we should certainly expect from the calculations of Table VIII.

It appears that 1 gm. of casein is completely dissolved at the temperature from 60° to 85°C. when it binds one-fifth of the amount of base necessary for its saturation at 20°C. At the temperature level from 21° to 37°C. the same amount requires one-third of the amount necessary for the saturation. Paracasein passes into solution when 1 gm. of this modification of casein binds one-half of the amount necessary to saturate an "unmodified" casein. The system casein-$Ca(OH)_2$ from 60° to 85°C. conforms to the last relation.

TABLE IX.

The Law of Multiple Proportion and the Properties of Casein as an Acid at Various Temperatures.

Temperature range °C. (1)	Base added (2)	Equivalent weight (3)	Base bound by 1 gm. of protein $1/(3) \times 10^{-5}$ (4)	138/(4) (5)	Whole numbers ascribed to (5) (6)
		gm.			
60–85	Monovalent	3700	27.0	5.1	5.0
21–37	" (25)	2100	47.5	2.9	3.0
60–88	$Ca(OH)_2$	1400	71.5	1.95	2.0
23–25	Paracasein-NaOH	1450	69.0	2.0	2.0
18–25	Maximum base-binding ("unmodified") NaOH (18)	725	138.0	1.0	1.0
" "	Maximum base-binding ("Nach Hammarsten") NaOH (18)	545	183.0	3/4	

Up to the present we have treated the system casein-$Ca(OH)_2$ as being identical with the system casein-monovalent base. The parallel is not strictly correct. A large body of experimental evidence indicates, as we have seen, that casein with $Ca(OH)_2$, at high temperature, forms a rather insoluble salt. If a similar change occurs in the properties of casein in this system, as it does in the system of casein-monovalent base, the relation of the combining weights (Table IX) to each other indicates that the solubility of casein in $Ca(OH)_2$ becomes independent of the temperature when about two-fifths of the salt formed is soluble in water.

The evidence gathered in this discussion seems to indicate that the base-binding capacity of casein is not only affected discontinuously by the temperature, but that there exists a definite multiple relationship associated with this discontinuous behavior, indicating that this change is of a stoichiometric nature.

SUMMARY.

1. The investigations dealing with the properties of casein as an acid were reviewed.

2. The solubility of uncombined casein in water was measured at 5°C. and found to be 0.70±0.1 mg. of N per 100 gm. of water.

3. Robertson's solubility measurements of casein in bases at various temperatures were recalculated and found to agree well with more recent measurements.

4. By combining the observations of several investigators, as well as the author's measurements of the solubility of casein, in base, at various temperatures, the following conclusions were reached:

(*a*) The solubility of casein in base is affected by the temperature in a discontinuous manner.

(*b*) There exist two ranges of temperature, one, extending from about 21° to 37°C. and the other from about 60° to 85°C. where the solubility of casein in base is practically independent of temperature.

(*c*) From 37° to 60° the equivalent combining weight of casein rises from the value 2100 to about 3700 gm.

5. By comparing the values of base bound by 1 gm. of casein at the two temperature ranges with a constant, the value of base necessary to saturate the same amount of casein, it was found that the latter value is a common multiple of the former values, indicating the stoichiometric nature of the effect of temperature.

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THE EFFECT OF RENNIN UPON CASEIN.

I. THE SOLUBILITY OF PARACASEIN IN SODIUM HYDROXIDE.

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I.

INTRODUCTION.

When small amounts of rennin, or of any proteolytic enzyme, are added to milk a clot appears. In the course of the clotting a modification occurs in the casein of milk, resulting in the production of a protein that has been called paracasein.[1]

The clotting of milk has been the subject of many investigations, and the explanations of the phenomenon that have been proposed may be classified, on the whole, into two groups. The first of these assumes that the clotting of milk is essentially an incipient proteolysis. The second interprets the reaction in terms of colloidal chemistry and assumes that no chemical modification has taken place, paracasein being only a physical modification of casein. According to this view, the change leading to the clotting of milk involves some colloidal properties of the casein.

Among the exponents of the theories falling under the first group was Hammarsten. As early as 1875 Hammarsten attempted to explain the action of rennin in terms of proteolysis, the casein being split into two parts: one, paracasein, and the other a whey albumin (1) Very similar views were held by Schmidt-Nielson (2) and by Slowtzoff (3). Van Herwerden advanced a modified theory. According to this, the resulting paracasein is made up of two substances, Paracasein *B* and Paracasein *C*. The latter (unlike Paracasein *B*) is not precipitated by acetic acid, and requires more concentrated $(NH_4)_2SO_4$ for its precipitation. It is soluble in water and gives a strong biuret reaction. After long action a proteose is formed which leads to the further decomposition of the molecule (4).

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[1] Casein for paracasein, and caseinogen for casein, according to the English classification.

The analytical identity of casein and paracasein has been the subject of several investigations. Köster (5), Rose and Schulze (6), Raudnitz (7), L. L. Van Slyke and Bosworth (8) and Bleyer and Seidl (9) analyzed paracasein. Harden and Macallum (10) found that the "conversion of caseinogen into casein by enzyme action is accompanied by the cleavage of N, P and Ca. Rennin action produces no soluble N or P. Trypsin splits off both soluble N and P. . . . . The cleavage products are specific for each enzyme and it is to this difference of enzyme action that the variation in behavior of the resulting casein is to be ascribed."

TABLE I.

The Elementary Composition of Paracasein.

Per cent of the element						Investigator
C	H	N	S	P	Ash	
52.79	6.98	15.84				Köster (1881)
53.94	7.14	15.14	1.01			Rose and Schulze (1885)
				0.66–0.84		Raudnitz (1903)
				0.85–0.87		Kikkoji (1909)
53.50	7.26	15.80	0.87	0.83	0.61	Van Slyke and Bosworth (1913)
53.50	7.26	15.80	0.72	0.71	0.07	Bosworth (1914)
53.47	7.19	15.78	0.72	0.71	0.09	" "
		15.64	0.81	0.83	0.1	Bleyer and Seidl (1922)
		15.70	0.76	0.80	0.085	" " " "
		15.65	0.78	0.79	0.09	" " " "
		15.60				" " " "
		15.61				" " " "
Average....53.45	7.17	15.65	0.81	0.78		
Casein......53.5–52.7	7.13–6.81	15.62–15.80	0.72–1.01	0.71–0.88		

Geake (11) investigated the elementary compositions of casein and paracasein and found them much alike.

Finally, Bosworth (12), using purified paracasein, found that the composition of paracasein was the same irrespective of the enzyme used to produce it. According to him, casein and paracasein have the same percentage composition. The results of these analyses are given in Table I. On the whole, paracasein does not differ much in elementary composition from casein.

We shall express our results in terms of nitrogen. In some of the calculations, however, it will be necessary to convert these values to terms of gm. of paracasein. In such calculations we shall use the average value for nitrogen obtained from Table I.

Whether a proteose is split off from the casein molecule by rennin has been a subject of much controversy. For this there seems to have been a twofold reason. Not all clots formed by rennin are alike. Their abundance and physical properties largely depend upon the Ca and H ion concentration of the milk. It follows that when the coagulation occurs, soluble nitrogen can be occluded and absorbed to different extents. Rennin preparations usually contain some proteolytic enzyme, and it has often been contended that rennin is itself a proteolytic enzyme. Therefore, if the proteolytic enzyme is not inactivated in the course of the coagulation by the products of the reaction, it will afterwards produce soluble nitrogen, and even P or S, depending upon the manner in which the complex protein molecule is split.

The study of the nature of the reaction between casein and rennin has been most successfully investigated by the study of the intermediary product of the reaction—the paracasein isolated from milk and subsequently purified. Van Slyke and Bosworth (13), using their methods for the purification of casein and for the purification of paracasein, found that the amount of base just necessary to hold paracasein in solution was almost exactly twice that necessary to dissolve casein. They concluded that:

> "the molecule of calcium caseinate containing four equivalents of base is split by rennin into two molecules of paracaseinate, each containing two equivalents of base. Such a paracaseinate is soluble in water, but insoluble in the presence of more than a trace of a soluble calcium salt. A molecule of calcium caseinate containing two equivalents of base is split by rennin into two molecules of paracaseinate, each containing one equivalent of base. Such a paracaseinate is insoluble in water."

Rennin therefore is not regarded by these investigators, strictly speaking, as a coagulating enzyme. Rather they consider the coagulation as a secondary effect, the result of a change in solubility. Further, they believe the action of rennin to be the first step in the proteolysis of casein (14).

Among the exponents of a purely physical point of view of the clotting of milk, Mellanby (15) must be mentioned. He supposed that the enzyme was adsorbed by the casein, thus forming the clot. Alexander (16) suggested that the casein in milk is protected by the lactalbumin; and that the enzyme destroys the lactalbumin, and thus permits the coalescence of the caseinogen particles. Schryver (17), after an extensive investigation of the solubility of casein subjected to various treatments, concluded that in milk:

> "the materials necessary for the clot formation pre-exist, but that aggregation formation is prevented by the adsorption of simpler molecules from the system. The conception was formed that a ferment, for which the colloidal substances could act as a substrate, could clear the surface of such substances of adsorbed bodies and thus allow aggregation (clot) formation to take place."

Recently, Wright (18) investigated the racemization curves of casein and paracasein and found them identical. From this fact he concluded that

> "rennet does not cause any proteolytic cleavage of the caseinogen molecule, but that coagulation is due to an alteration of the colloid state of the caseinogen by which precipitation in the presence of bivalent metal ions is facilitated."

The present investigation was undertaken on the basis of the simplest chemical hypotheses. In order to know the nature of a reaction, one must know the chemical properties, not only of the reactants, but also of the products. The reactant, casein, is well known because of the extensive investigations of Lacqueur and Sackur, T. B. Robertson, L. L. Van Slyke, E. J. Cohn and their respective coworkers. We propose therefore to study paracasein: its physicochemical properties and behavior. In this we hope to identify paracasein as a chemical substance, in the belief that a more complete knowledge of the reactants and of the end-products of reactions promoted by enzymes may give more reliable information regarding their nature, and perhaps also their mechanism.

II.

The Preparation of Paracasein.

Six different paracasein preparations have been used in this investigation. Of these the protein was in five cases precipitated from milk by active preparations of rennin, and in one by pepsin. The methods used for the purification of the paracasein were much like those already described by L. L. Van Slyke and Baker (19). A modification of their method (20) yielded reproducible results with casein and with paracasein preparations. Since in the latter case the first precipitation was affected by the enzyme, it was in some cases unnecessary to add any acid. In this only did the method of purification of casein differ from that of paracasein. In one of the paracasein preparations we used a large excess of alkali, in order to study its effect on the protein. No appreciable differences were found between preparations treated with an amount of alkali just necessary to dissolve them and a preparation in which an excess of alkali had been used.

Paracasein I.—An active rennin powder prepared by the Digestive Ferments Company was used in the preparation of Paracasein I. To 7 liters of skimmed milk was added 0.001 gm. of the powder. The milk was then kept for 24 hours at 25°C. under an excess of toluene. After the separation of the clot the precipitate was washed seven times with four times its volume of distilled water. The precipitate was brought into intimate contact with the successive wash waters by means of a motor-driven, glass, screw-shaped stirrer. On each washing the reaction of the the clot appeared to be more acid, until by the seventh washing the pH had become 4.6. This reaction is the optimum for the precipitation of casein (21). The change of the reaction of the precipitate can most easily be accounted for in terms of the Donnan equilibrium; for, since both calcium paracaseinate and uncombined paracasein are relatively insoluble, it may be conceived that transformation of the former into the latter takes place by the diffusion of calcium ions from the casein clot, and their replacement by hydrogen ions. This case is thus comparable with the Donnan theory of hydrolysis through a membrane. It is of interest to note that this phenomenon did not take place with all of our preparations.

After the clot was washed, the paracasein was dissolved by the addition of 0.1 N sodium hydroxide. The reaction of the resulting solution was found to be approximately pH 7.0. The solution was passed through a Sharples centrifuge, and then filtered through filter paper pulp. The paracasein was reprecipitated by means of 0.1 N hydrochloric acid, delivered very slowly from a capillary tip extending well into the solution. The solution was continuously and rapidly stirred. The acid was added until the pH was 4.6, which was found to be the point at which paracasein flocculates the best. Finally, the precipitate was washed with water, redissolved by sodium hydroxide, reprecipitated by hydrochloric acid and again washed until chloride-free. The whole preparation was carried on in the manner that has already been described in detail for casein (20).

Paracasein II.—Pepsin powder prepared by the Parke, Davis and Company was used in the preparation of Paracasein II. To 4 liters of milk was added 0.002 gm. of the powder. The subsequent washing and purification was carried on in the same way as for Paracasein I.

Paracasein III.—Active rennin powder prepared by the Digestive Ferments Company was used in the preparation of Paracasein III. To 3½ liters of milk was added 0.005 gm. of the powder. The clot was washed four times as before, and enough 0.1 N sodium hydroxide was added to bring the solution to pH 9.0. The subsequent purification was carried on in the same way as in the cases of the Paracaseins I and II.

Paracasein IV.—3 liters of milk were warmed to 32°C. Immediately 0.036 gm. of Hansen's salt-free active rennin powder was added to it. The milk was vigorously stirred. After an elapse of 1¼ hours, the milk coagulated. It was immediately cooled down to 5°C., and extensively washed with water.

Otherwise the procedures of purification were the same as in Preparations I, II and III.

Paracasein V.—In this preparation, we used the Morgenroth method of coagulation of milk. It is based on the following observation: Milk to which rennin is added and which is kept at a low temperature does not coagulate, although the transformation of casein into paracasein presumably takes place. This milk can be coagulated after a short exposure to high temperature. The usual explanation of this phenomenon is that paracasein, even in the presence of salts, does not coagulate at a low temperature. The coagulation may be brought about by warming the milk.

3 liters of milk were cooled to about 5°C., and 0.01 gm. of Hansen's salt free rennin preparation was added to it. The milk was placed in a cold room at 5°C., and left undisturbed for 20 hours. No coagulation took place at that temperature. The milk was then warmed to 35°C., which brought about its coagulation in a time slightly less than 30 minutes. After the coagulation took place the milk was cooled to 5°C., and purified by the ways described.

Paracasein VI.—To 3 liters of milk we added, in this preparation, 0.03 gm. of considerably weakened Hansen's salt-free rennin preparation. After an elapse of about 12 hours at about 18°C. the milk coagulated.

The subsequent purification of this preparation of paracasein was carried on in the usual way.

Since, in the preparations of paracasein, an enzyme was added to the systems, they were no longer comparable to systems containing only casein. If, however, the enzyme added were wholly inactivated during the course of the purification, the investigation of paracasein would become comparable with that of casein.

Our pepsin preparation had roughly twice as much enzyme as the rennin preparations, both in respect to coagulative and proteolytic activity. Therefore, Paracasein II had about ten times as much enzyme as Paracasein I. The same relation is true of Paracaseins III and I. But, Paracasein III was also brought to an alkaline reaction in the course of its precipitation, to pH 9.0. It is well known that both pepsin and rennin are very sensitive to alkali, and experiments carried out in this laboratory upon the latter confirm the observation that this enzyme is quickly inactivated even at neutral reactions. We conclude from this that after purification Paracasein III should contain less enzyme than Paracasein II.

If the property of prepared paracasein were dependent upon the proteolysis produced by rennin or pepsin added, we should expect that Paracasein II, when equilibrated with a certain amount of sodium hydroxide, would produce much more soluble nitrogen than, for example, Paracasein I or III. This was found not to be the case. Preparations of paracaseins differed but slightly from each other, and their solubilities were practically independent of time.

As we shall see from one of the next sections, the solubility of paracasein can be measured both at low and at high temperature. If the solubility is due, even in part, to a proteolytic enzyme, the rise in temperature would increase the solubility. On the contrary, the temperature coefficient of the binding capacity of paracasein with NaOH does not differ at all from the corresponding temperature coefficient of casein.

Therefore, we are inclined to think that in the course of the purification of paracasein the enzyme added was wholly inactivated, and did not interfere appreciably with subsequent measurements.

III.

The Solubility of Paracasein in Water.

Paracasein, purified to the same extent as casein and chloride-free, still contains large amounts of caseose which appear in the solution, after a thorough washing of the suspension. The amount of soluble nitrogen varied slightly from preparation to preparation, and the pH approached 6.5, presumably as a result of the presence of these substances. If we assume that paracasein is soluble in water, the nitrogen found in the filtrate may be represented as being composed of (1) nitrogen arising from the solution of paracasein in water and (2) nitrogen arising from any other soluble substance present in our system. In order to simplify our reasoning, let us consider first a simpler chemical case. Let us assume that we have in a liter of water a large excess of barium sulfate, together with some magnesium sulfate and sodium sulfate, and that we wish to measure the solubility of barium sulfate in water. Let our method for the estimation of barium sulfate be limited to the measurement of the SO_4 radical only. Should one equilibrate such a mixture and then filter some of it and estimate the gm. of SO_4 present in the filtrate, values for the solubility of barium sulfate, greater than the theoretical solubility, would be obtained. The magnitude of this increment will of course depend upon the amount of more soluble sulfate salts present. An attempt, however, to wash such a system systematically, will result in values ranging closer and closer to the value of the solubility in water of the least soluble substance, barium sulfate in our case. Several criteria for the value of the solubility of barium sulfate in water might be applied. First, it should be independent of the way in which barium sulfate was prepared; in the second place, its solubility should be independent of the length of time the solvent is equilibrated with the saturating body, which should finally dissolve in successive fractions of the same solvent to the same characteritic extent, since a chemically pure substance has always the same physicochemical properties.

The situation with paracasein was very like that with the inorganic analog we have just considered. In our case paracasein, a substance of unknown solubility, was obtained with a mixture of other nitrogen-yielding substances, also of unknown solubility. The method used to obtain a pure substance from this mixture was identical with that described for the purification of barium sulfate.

The experimental procedure was as follows: Samples of Paracaseins I, II and III were placed in three Pyrex bottles. They were diluted with water to about ten times the volumes of the precipitates. The suspensions were stirred by means of a motor-driven, glass, screw-shaped stirrer at about 7°C. The stirring did not change appreciably the state of subdivision of the paracasein precipitate. Only a few hours were required to bring the suspension to the state of equilibrium with the watery phase. The time of stirring was, however, extended to 48 hours and often to more. After each stirring the supernatant liquid was decanted and part of it filtered through a No. 42 Whatman filter. The nitrogens were determined on 50 cc. aliquots by the Kjeldahl method. On the same filtrate a colorimetric pH measurement was taken. Then the bottles were refilled with cold water, and the operation repeated. The results of the experiment are tabulated in Table II.

The results of the experiment show a constancy of the solubility of paracasein in water, when freed from hydrolytic products. Only one measurement, namely, the solubility of Paracasein III on the seventh washing, diverges by a value greater than the experimental error.

We have reason to believe that this preparation became contaminated at this stage of washing. It was probably for this reason that the solubility of this preparation was slightly higher.

There is one more bit of interesting evidence that the system of paracasein and hydrolytic products is very similar to the system of our inorganic analog. Paracasein II, after the first and the third washings (Table II), was stirred for 5 and 10 hours, respectively, and then for 48 and 24 hours. The amount of nitrogen in the liquid phase remained constant. This evidently indicated that the soluble nitrogen did not arise from the activity of a proteolytic enzyme, since then, with time, the amount of protein hydrolyzed should increase. This was not the case. Our systen was a mixture of two or more substances differing in their solubility in water.

TABLE II.

The Solubility and the Hydrogen Ion Activity of Paracasein in Water.

Time of stirring	No. of washings	Solubility: mg. N in 25 cc. Paracasein preparation			pH Paracasein preparation		
		I	II	III	I	II	III
hrs.							
5	1		1.37			6.5	
53	1		1.37			6.5	
10	2		0.77				
10	3		0.48			6.4	
34	3		0.47				
12	4	0.35	0.33	0.41	6.4	6.4	6.4
48	5	0.14	0.16	0.17	6.4	5.25	6.2
48	6	0.14	0.15	0.20	5.25	5.25	5.25
72	7	0.15	0.17	0.25	5.1	5.2	5.25
48	8		0.15				
144	8	0.16					

The average of all the determinations from the fifth down to the eighth washing gave the value of 0.17 ± 0.03 mg. of nitrogen in 25 cc. The solubility of paracasein per 1000 gm. of water is therefore equal to 6.8 ± 1.2 mg. of nitrogen, at about 7°C.

The pH of a purified paracasein is most probably 5.2. It is more alkaline than the pH at which paracasein is best precipitated, which is in the neighborhood of pH 4.6.

In an investigation of the effect of temperature upon the solubility of casein in water (22), we found that this protein dissolves to an extent of 7.0 ± 1.0 mg. protein N in 1000 gm. of water at 5°C. Comparing this solubility with the one obtained for paracasein, it is evident that the solubilities of these two proteins are identical at practically the same temperature. We may conclude that paracasein, at low temperature, dissolves in water approximately to the same extent as casein.

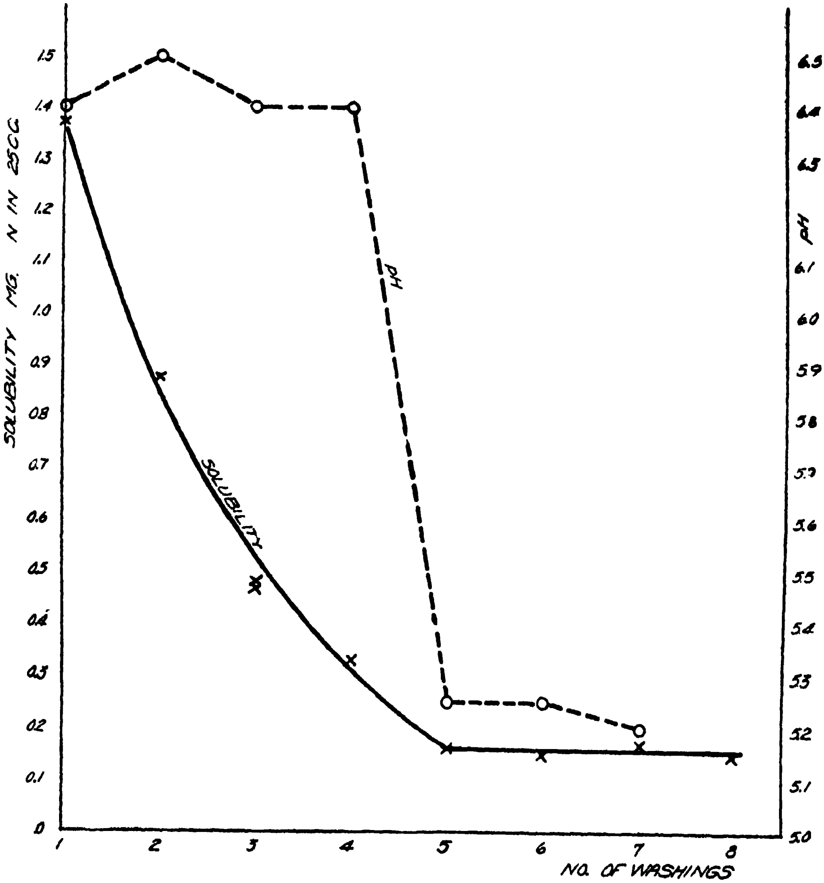

FIG. 1. The purification of paracasein from hydrolytic products and its solubility in water in an uncombined state.

IV.

The Solubility of Paracasein in Sodium Hydroxide.

Similarly to casein, when sodium hydroxide is added to paracasein, this protein forms a sodium compound which is soluble in water. By estimating the amount of paracasein dissolved by a given amount of sodium hydroxide, we can measure the combining capacity of this protein as an acid, provided all the base is bound by the protein in solution.

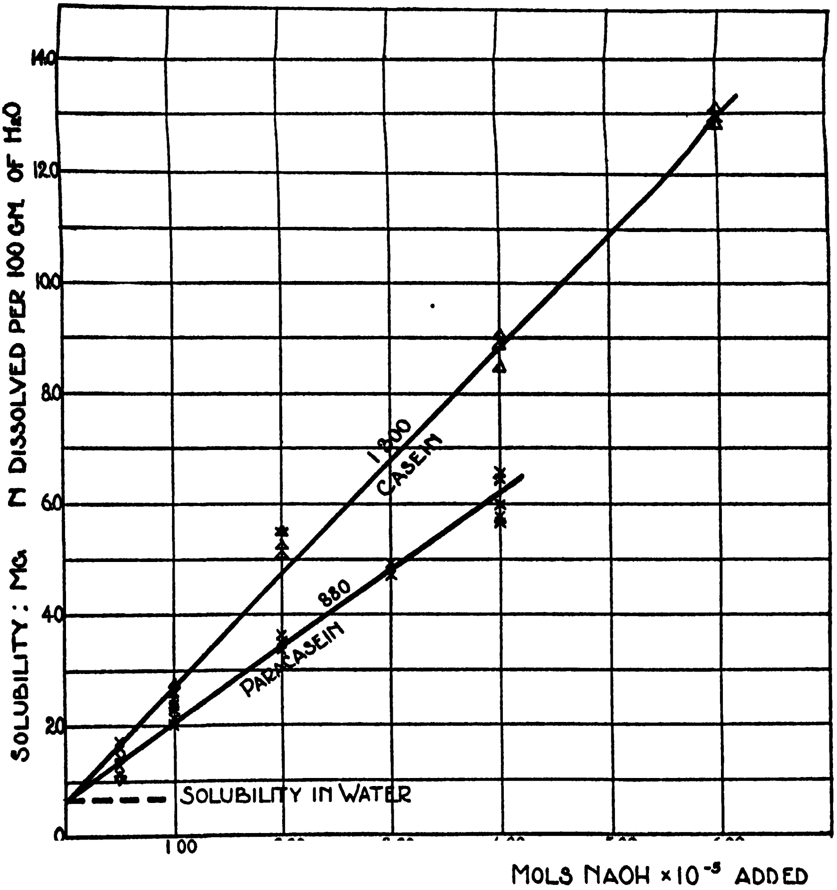

FIG 2. The solubility of paracasein in NaOH at 5°C. as compared with the solubility of casein at the same temperature (22).

Before undertaking the actual measurements of the solubility of the protein, it seems to be advisable to determine the time in which this system will come to equilibrium. A sufficient criterion for this state for an isothermal, isopiestic system is the independence from time of the amount of protein dissolved by a given amount of base.

In order to elucidate this problem, we undertook the following experiment. Small amounts of Paracaseins II and III, in suspension, used in the experiments on solubility of these proteins in water, were pipetted out into 100 cc. volumetric flasks. To these flasks were then added small amounts of sodium hydroxide and carbon dioxide-free water until the volume of the solution in each reached the 100 cc. mark. The flasks were then placed in a shaking machine and equilibrated for various periods of time at about 5°C. The contents of the flasks were then filtered through No. 42 Whatman paper filters and the filtrates analyzed for nitrogen by the Kjeldahl method.

TABLE III.

The Solubility of Paracasein in NaOH as a Function of Time.
Temperature: 5.0° ± 0.5°C.

Paracasein preparation	NaOH added: mols $\times 10^{-4}$	Solubility: mg. N in 20 cc. of filtrate after an elapse of		
		1 hr.	3 hrs.	6 hrs.
(1)	(2)	(3)	(4)	(5)
II	1.00	0.52	0.52	0.54
III	0.50	0.23	0.23	0.24

The method used in this investigation was very similar to the one described by E. J. Cohn and Hendry (20) in their investigation upon the solubility of casein. The results of this experiment are given in Table III. They indicate, on the whole, that the protein dissolves readily in sodium hydroxide. The solubility of this protein is practically independent of time.

The investigation of the solubility of paracaseins in sodium hydroxide was then undertaken. The method for the determination of soluble nitrogen was identical to that one already described. The time of equilibration was varied from 2 to 24 hours. The temperature at which the experiments were carried out was 5.0° ± 0.5° C. and 23.0° ± 2°C.

The amount of the saturating body was varied within large limits without any appreciable effect upon the solubility of paracasein.

TABLE IV.

The Solubility of Paracasein in Sodium Hydroxide at 5.0° ± 0.5°C.

NaOH added: mols × 10^{-5}	Experiment No.	Paracasein preparation		
		I	II	III
		Mg. N dissolved per 100 gm. of water		
(1)	(2)	(3)	(4)	(5)
0.50	4 5 1	1.4 1.4	1.6 1.7	1.1 1.3
1.00	2 1 2	2.4 2.3	2.6 2.6	2.0 2.1
	4 2	2.3 2.4	2.6 2.7	
	3		2.4 2.4	
	5		2.4 2.6	
2.00	3 2 2	3.4 3.5	3.5 3.6	3.5
	4 5	3.4	3.4 3.4	
3.00	5		4.7 4.9	
4.00	3 5	5.65 5.75	6.45 6.55	
	4	5.65 6.00		

Paracasein is much like casein in this respect: the solubility with sodium hydroxide is independent of the amount of the protein in the precipitate, and is solely determined by the amount of the base added.

The results of these investigations are recorded in Tables IV and V, and Fig. 2.

The solubility of paracasein at 5°C. in NaOH (Fig. 2), unlike its solubility in water, is distinctly different from that of casein. An equal amount of base carries less paracasein into solution than it does casein. About 810 gm. of paracasein are carried into solution by 1 mol of NaOH.

TABLE V.

The Solubility of Paracasein in Sodium Hydroxide at 23° ± 2°C.

Paracasein preparation (1)	Amount of NaOH added: mols × 10^{-5} (2)	Solubility: mg. N dissolved per 100 gm. of water (3)	Equivalent weight: gm. of paracasein (4)
I	2.00	3.9	
	6.00	12.9	1450
II	0.50	(1.8)	
	1.00	3.3	
	5.00	11.9	1400
II	2.00	4.9	
	6.00	13.5	1400
IV	2.00	3.65	
	6.00	13.2	1550
V	2.00	5.35	
	4.00	10.1	1500
Average............	....	....	1450
VI	2.00	5.17	
	4.00	10.55	1700

We have repeated these measurements at a temperature of 23° ± 2°C. In these investigations we have included Paracasein Preparations I and II, as well as IV, V and VI.

The results of these measurements are recorded in Table V. As in the measurements at 5°C., paracasein is distinctly different from casein at 23° ± 2°C.: its average equivalent is 1450, while casein in the corresponding range of temperature had the equivalent weight of 2100 ± 100 gm. (20).

The equivalent weight of Paracasein Preparation VI, which was prepared by the use of a considerably weakened rennin preparation, is outside our experimental error. The equivalent weight of this preparation is about 1700 (Table V). We shall consider this paracasein preparation in the following communication.

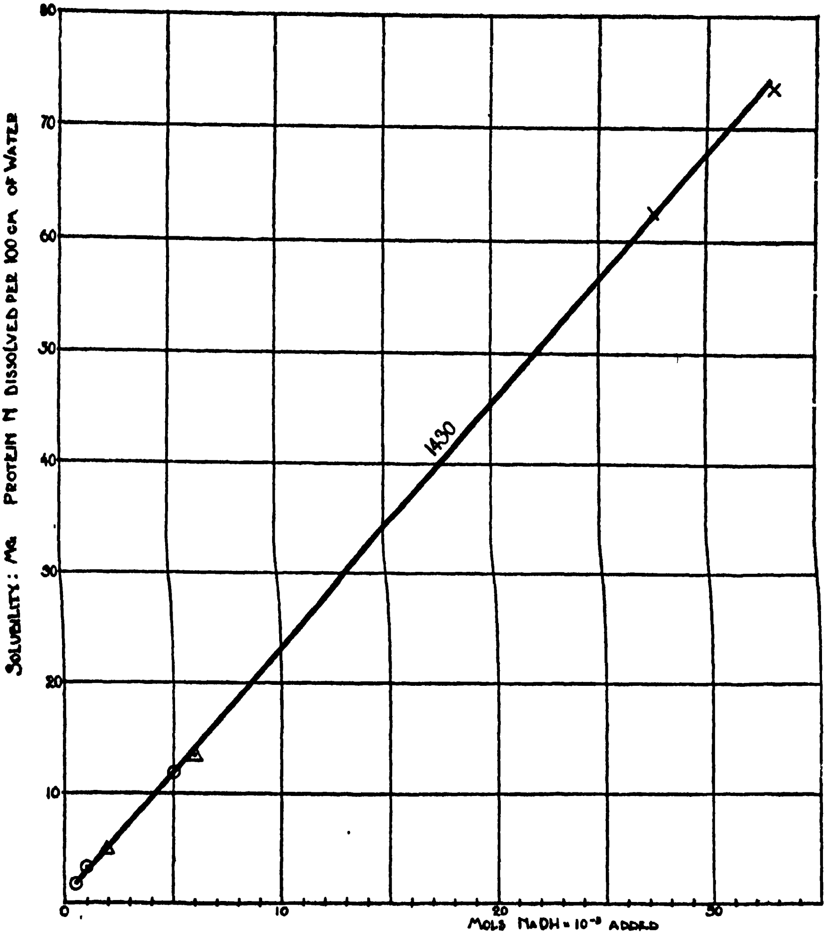

FIG. 3. The solubility of Paracasein Preparation II in small and large amounts of NaOH at 23° ± 2°C.

In all of these investigations, we have confined ourselves to estimates of the solubility of paracasein with small amounts of sodium hydroxide. It is of interest whether paracasein, like casein (20) at 25°C., displays a constant base-combining capacity independent of the amount of base added. For this purpose we made solubility measurements upon Paracasein Preparation II with large amounts of NaOH, at 25°C. They are recorded in Fig. 3. The suspensions contained 86.0 mg. of paracasein N. With 32.9×10^{-5} mols of NaOH, 73.4 mg. N passed into solution. About 85 per cent of the protein was therefore dissolved. From the calculation of the equivalent weights of Fig. 3, it becomes evident that the solubility of Paracasein II is independent of the amount of base added. Like casein, this paracasein behaves toward NaOH at 25°C. as a homogeneous body; at least the 85 per cent of precipitate that dissolved appeared to be homogeneous.

V.

CONCLUSIONS.

The investigation was carried out upon a substance referred to as paracasein. It is not improbable that one can obtain from the clot produced by rennin, substances of diverse properties. Therefore, it is of importance to define our paracasein in rigorous terms. We shall refer to paracasein as a modification of casein produced in the clotting of milk by rennin or pepsin preparations, being redissolved several times by NaOH and possibly further acted upon by the enzyme; then washed at the maximum flocculation point until the product displays a constant solubility in water, independent of time and of the number of washings.

In studying the physicochemical properties of such a substance, it is of extreme importance to know whether the system contains any free enzymes.

The absence of the proteolytic enzyme from our parcasein preparation was concluded from the following observations: (1) The amount of enzyme originally added to the milk has no bearing upon the solubility of the product in water or NaOH. (2) The time factor has no effect upon the solubility of paracasein in water. (3) The solubility with sodium hydroxide is practically independent of time. (4) The temperature coefficients of the solubilities of casein and paracasein in sodium hydroxide are identical (Table VI). To our mind, these criteria are sufficient to conclude that no active proteolytic enzyme was present in our purified paracasein preparations.

Paracasein dissolves in water to an extent of about 7.0 mg. of N per 1000 gm. of water at 7°C., which within the experimental error is identical with the solubility of casein at about the same temperature, since the properties of paracasein as an acid are distinctly different from those of casein, one may conclude that, in some cases, one can change the acid properties of a protein without affecting its solubility in water.

TABLE VI.

A Comparison of the Acid Properties of Paracasein and Casein.

Solubility range: mols NaOH × 10^{-3} (1)	Temperature, °C. (2)	Equivalent weight: gm. of protein		Ratio (3)/(4) (5)
		Casein (3)	Paracasein (4)	
0–6.00	5.0	1300		1.48
0–4.00	5.0		880	
Any	21–37	2100		1.45
"	21–25		1450	

As we have already pointed out, paracasein is distinctly different from casein in its capacity to bind base, both at 5° and at 23° ± 2°C.

At 5°C., paracasein, in most of the solubility regions investigated, dissolves at a rate of 810 gm. of the protein per 1 mol of NaOH. At the corresponding temperature and solubility range, casein dissolves to the extent of 1300 gm. per 1 mol of NaOH. At the temperature level of 21°–25°C., the corresponding figures are 1450 gm. for paracasein and 2100 gm. for casein.

This information is compiled in Table VI. In column (5) of this table is given the ratio of the equivalent weight of casein to the equivalent weight of paracasein. The relation seems to yield a constant, which indicates that the temperature coefficients of the solubility of paracasein and casein in NaOH are identical.

As the calculation of Table VI shows, paracasein is nearly 1.5 times more acid than casein, the capacities of casein and paracasein to bind base standing to one another as the whole numbers 2 and 3 (22).

The fact that in using varying amounts of the enzyme one obtains practically identical parcaseins indicates that most of the paracaseins obtained in this investigation are final products of a reaction promoted or brought about by rennin or pepsin.

The information gathered in this research substantiates the conclusion reached by several investigators, that the transformation of casein into paracasein is of a chemical nature.

VI.

SUMMARY.

1. The preparation and purification of paracasein was described and certain criteria for the absence of free enzyme provided for.

2. The solubility of purified paracasein in water at low temperature was studied, and found practically identical with the solubility of casein.

3. The capacity of paracasein to bind base was investigated by means of its solubility in NaOH at 5° and at 23° ± 2°C., and found to be distinctly different from that of casein.

4. At these two temperature levels paracasein had a 1.5 greater capacity to bind base than casein. The equivalent combining weights of paracasein and casein were found to stand each to the other, apapproximately, as 2 to 3.

5. This relationship suggested that the temperature coefficients of the solubility of paracasein and casein in NaOH are identical.

6. This evidence indicates that paracasein is a modification of casein, distinguishable by physicochemical means.

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